DOES NOT CIRCULATE

VOL. XXXVI. No. 4] [AUGUST, 1955

UNIVERSITY OF MICHIGAN
SEP 14 1955
MEDICAL LIBRARY

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

EDITOR
L. P. Garrod

ASSISTANT EDITORS
C. F. Barwell, H. Lehmann, R. A. Shooter

ADVISORY EDITORS
M. P. Curwen, J. R. Marrack, A. A. Miles, W. T. J. Morgan, C. G. Pope
R. W. Scarff, Wilson Smith, D. D. Woods

with the support of

S. P. Bedson, E. C. Dodds, C. L. Evans, H. W. Florey, H. D. Kay,
J. W. McLeod, W. J. Tulloch

DIRECTOR
Paul Fildes

LONDON
H. K. LEWIS & Co. Ltd.
136 GOWER ST., W.C. 1

on behalf of

'The British Journal of Experimental Pathology.' Registered Office: 120 Moorgate, London, E.C. 2

Annual Subscription, January to December, post free, Four Pounds net
Single Numbers, Sixteen Shillings net

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal was founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, pharmacological, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

Forwarding Papers and Correspondence.—Papers should be forwarded to Professor L. P. Garrod, Department of Pathology, St. Bartholomew's Hospital, London, E.C. 1.

General Correspondence relating to the conduct of the Journal should be addressed to Sir Paul Fildes, Sir William Dunn School of Pathology, University of Oxford.

Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C. 1.

Date of Publication.—The Journal is published bi-monthly, in the middle of February, April, June, August, October, and December.

Rapidity of Publication.—Every endeavour is made to publish papers as soon as possible, but under present conditions a delay of 4–6 months between receipt and publication can rarely be avoided.

Proofs.—Proofs will in future be submitted to authors in any part of the world. Prompt return by air mail from distant contributors is necessary if publication is not to be delayed.

Reprints.—25 reprints will be supplied free to authors who are registered as personal subscribers to the Journal. Additional copies must be ordered from the Publishers *before the issue is printed.*

Estimates for reprints will be supplied on request to the Publishers.

Made and printed in Great Britain.

Vol. XXXVI, No. 3 (June, 1955), was issued on 11.vii.55.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XXXVI AUGUST, 1955 NO. 4

MINERAL REQUIREMENTS FOR GROWTH OF *STAPHYLOCOCCUS PYOGENES*. EFFECT OF MAGNESIUM AND CALCIUM IONS.

R. A. SHOOTER AND H. V. WYATT.

From the Department of Bacteriology, St. Bartholomew's Hospital, London, E.C.1.

Received for publication March 18, 1955.

MUCH attention has been paid to the action of inorganic ions on bacterial enzyme systems. Relatively little, however, is known of the mineral requirements of bacteria for growth. Webb (1951) has shown that for many bacteria magnesium is needed for cell division, and Henry and Stacey (1946) have shown that it is required for the formation of the Gram staining complex. Burton, Campbell and Eagles (1948) and King, Campbell and Eagles (1948) have defined the requirements of *Pseudomonas pyocyanea* for growth and the formation of pigments, and Shankar and Bard (1952) have demonstrated that *Clostridium welchii* needs sodium, potassium, magnesium, calcium and iron.

For the study of bacterial growth and toxin production in media of known composition, and perhaps of the action of antibiotics, a knowledge of inorganic ion requirements is desirable. This paper records the first stage of an investigation of the mineral requirements of *Staphylococcus pyogenes* using an ion exchange resin to remove cations from a complex natural medium.

MATERIALS AND METHODS.

Cleaning of glassware.

Test tubes were first washed with hot tap water and scouring powder. After repeated rinsing with metal-distilled water they were autoclaved inverted in metal-distilled water, and then re-autoclaved in Pyrex-distilled water. Other glassware was cleaned with potassium dicromate-sulphuric acid, washed, rinsed and then autoclaved with metal-distilled water and finally re-autoclaved with Pyrex-distilled water.

Reagents.

Except for pipettes, Pyrex glassware was used. Water used for the preparation of stock solutions, dilutions of reagents and inocula, and for the final autoclaving of glassware was metal-distilled water re-distilled in Pyrex glass. Amberlite IRC-50 was kindly supplied by Rohm and Haas of Philadelphia. The stock solution of Ca^{2+} was made by dissolving $CaCO_3$ in a calculated volume of conc. HCl and diluting. Mg^{2+} was added as $MgSO_4.7H_20$, K^+ as KCl and Na^+ as NaCl. With the exception of arginine, other chemicals used were of A.R. grade. Flame photometry detected no K^+ in the arginine.

Medium.

The medium was a 1 per cent solution of Lab Lemco (Oxo Ltd.) with 1 per cent peptone (Evans) and 0·5 per cent glucose, and was prepared in batches of 3 or 4 l. After removal of phosphates the pH was adjusted to 7·3, and 1 l. was kept as control untreated broth. The remainder was divided into two portions, and each run down a 1·1 cm. diameter glass tube filled for a length of 140 cm. with IRC-50 which had been converted to the Na^+ cycle by treatment with $NaHCO_3$. The resin was not buffered: after passage through it, the broth was adjusted to pH 7·3 with HCl. Both control and treated broth were sterilised by autoclaving at 10 lb. per sq. in. (703 g. per sq. cm.) for 30 min.

The control broth contained approximately 2 mg. ions/l. Mg^{2+}, 0·3 mg. ions/l. Ca^{2+}, 20 mg. ions/l. K^+ and 18 mg. ions/l. Na^+. After treatment Mg^{2+} was reduced to less than 0·04 mg. ions/l., Ca^{2+} to approximately 0·015 mg. ions/l., K^+ to approximately 0·075 mg. ions/l., while Na^+, for which the other cations were exchanged, was increased to approximately 87 mg. ions/l. We are indebted to Dr. H. E. Archer for the estimation of Ca^{2+}: Na^+ and K^+ were determined with a flame photometer; Mg^{2+} was determined using Titan Yellow. Paper chromatography, for which we are indebted to Dr. G. H. Apthorp and to the Research Laboratories of Oxo Ltd., showed the control medium to contain free amino acids including arginine. After passage through the resin the medium contained the amino acids originally present except that free arginine was reduced to a trace. Combined arginine was present in the treated medium as shown by the Sakaguchi test and in the control medium as shown by chromatography of the components after acid hydrolysis.

Agar used in the preparation of solid media was treated by a modification of the method described by Webb (1949). New Zealand agar (25 g.) was washed with 2 l. of 0·1 N-HCl. The agar was then rinsed with 4 l. of Pyrex-distilled water, and the pH adjusted to 7·3. For use 2 g. of agar were added to 80 ml. of broth and the total volume made up to 100 ml. with water and stock solutions.

Preparation of cultures.

In most of this work a coagulase-positive strain (Terry) of *Staph. pyogenes* was used. For experiments in fluid medium 4 ml. of broth were made up to 5 ml. by the addition of necessary reagents and water. The standard inoculum was 1 drop of a mixture of 2 drops of a 5-hr. broth culture (made from an 18-hr. broth culture) in 5 ml. of water. A Pasteur pipette of 0·04 in. (0·102 cm.) external diameter was used for dropping, and the inoculum contained 10,000 to 50,000 bacteria. Cultures were incubated at 37°, tubes being covered with glass caps. After incubation 5 drops of 40 per cent formalin were placed in each tube and growth estimated using a Spekker absorptiometer with H508 filters and 1 cm. cells. Each reading recorded is the average of the readings of three or more cultures.

RESULTS.

In all these results growth in experimental tubes is expressed as a percentage of 18-hr. growth in control tubes containing untreated medium of the same batch as the experimental medium, inoculated at the same time with identical inocula. Absorptiometer readings at 18 hr. for the control medium varied between 0·650 and 0·800 according to the staphylococcal strain used. Each batch of treated medium failed to support the growth of *Staph. pyogenes* and no growth could be detected with the absorptiometer at 18 hr. after the addition of Mg^{2+} at 0·008 mg. ions/l., Ca^{2+} at 0·0025 mg. ions/l., or K^+ at 0·25 to 50 mg. ions/l. separately.

Effect of magnesium on growth.

Growth with added Mg^{2+} is shown in Fig. 1. At 18 hr. maximal growth was reached with 0·2 mg. ions/l., but this was approximately 45 per cent of the control growth. By adding a constant amount of arginine at varying Mg^{2+} concentrations, growth was initiated with less Mg^{2+} than when Mg^{2+} was used alone, and an overall increase in growth was obtained up to a Mg^{2+} concentration of 0·4 mg.

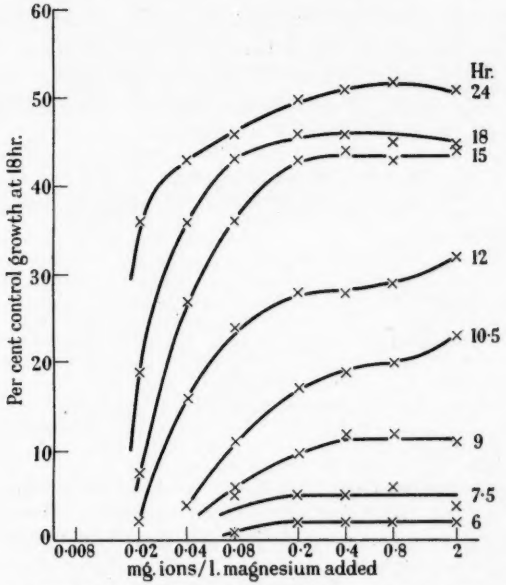


FIG. 1.—Effect of the addition of Mg^{2+} on the growth of *Staph. pyogenes* between 6–24 hr. (Broth batch L.1.)

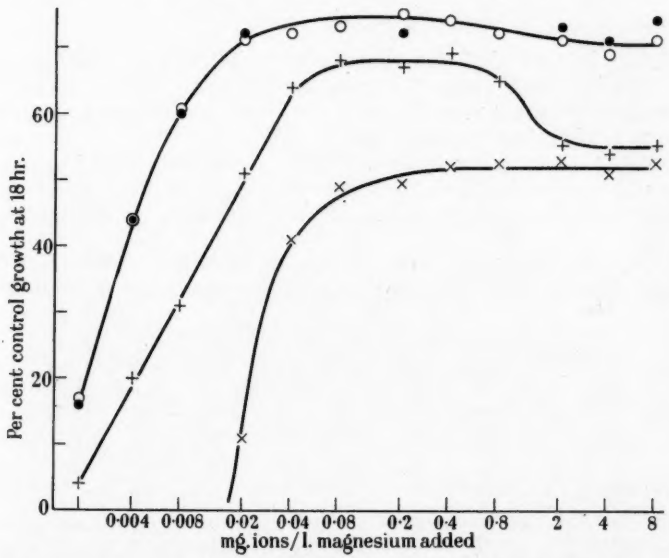


FIG. 2.—Effect on the growth of *Staph. pyogenes* at 18 hr. of the addition of Mg^{2+}, Mg^{2+} and arginine, Mg^{2+} and Ca^{2+}, and Mg^{2+}, Ca^{2+} and arginine. (Broth batch L.2.) × Mg^{2+}. + Mg^{2+} and 0·0005 M-arginine. ○ Mg^{2+} and 0·05 mg. ions/l. Ca^{2+}. ● Mg^{2+}, 0·05 mg. ions/l. Ca^{2+} and 0·0005 M-arginine.

ions/l. (Fig. 2). Between 0·4 and 2 mg. ions/l. this increase diminished, and above 2 mg. ions/l. of Mg^{2+} arginine had no effect on the amount of growth. That this was not the result of a simple relationship between Mg^{2+} and arginine is suggested by Table I which shows that varying the concentration of arginine had no appreciable effect at 2 mg. ions/l. Mg^{2+}.

TABLE I.—*Effect on the Growth of* Staph. pyogenes *at* 18 *hr. of Varying Concentrations of Arginine in the Presence of* Mg^{2+}. *Results Expressed as a Percentage of Control Growth.*

Magnesium.					
0·8 mg.ions/l.		2 mg.ions/l.			
—	0·0005-M arginine.	—	0·00025-M arginine.	0·0005-M arginine.	0·001-M arginine.
52	65	53	51·5	55·5	57

Broth batch L.2.

Effect of calcium on growth.

Growth, though less than that with Mg^{2+}, occurred when Ca^{2+} only was added to the medium (Fig. 3). This effect was observed with as little as 0·005 mg. ions/l. and increased slowly with increasing Ca^{2+} up to 2·5 mg. ions/l. The greater growth with higher concentrations of Ca^{2+} may be due to the presence of impurities. The addition of arginine increased growth at the lowest calcium levels, but had no effect at and above 0·05 mg. ions/l. of Ca^{2+} (Fig. 3).

Effect of magnesium and calcium on growth.

While both Mg^{2+} and Ca^{2+} stimulated growth singly, the two elements together were more effective and Table II shows the results of adding them alone and in combination. Optimal growth under the latter conditions was reached with 0·02 mg. ions/l. Mg^{2+} and 0·05 mg. ions/l. Ca^{2+}. Between 0·02 to 4 mg. ions/l. Mg^{2+} and 0·025 to 2·5 mg. ions/l. Ca^{2+} growth ranged from 65 to 75 per cent, with an average of 70 per cent of the control growth (Fig. 2 and 3). The level of growth was thus constant over a wide range of Ca^{2+} and Mg^{2+} concentrations, and as

TABLE II.—*Effect of Varying Concentrations of* Mg^{2+} *and* Ca^{2+} *on the Growth of* Staph. pyogenes *at* 18 *hr. Results Expressed as a Percentage of Control Growth.*

Magnesium mg.ions/l. added.	Calcium mg.ions/l. added.										
	—	0·0025.	0·005.	0·025.	0·05.	0·125.	0·25.	0·375.	0·5.	1·25.	2·5.
—	0	0	7	12·5	17	17	18	19·5	19·5	23	35
0·004	0	20	29	40	44	41	40	42	44·5	—	—
0·008	0	33·5	42	54	60·5	58	59	59	59	—	—
0·02	11	—	59	69	71	69	65	69	67	—	—
0·04	41	—	66	66	72	68	69	69	71	66	69
0·08	49	—	66	70	73	72	70	72	67	70	70
0·2	49·5	—	66	67	75	69	73	71	72	—	—
0·4	52	—	65	67	74	72	70	69	69	—	—
0·8	52·5	—	66	71	72	68	72	70	74	—	—
2	53	—	—	—	71	—	—	—	70	71	70
4	51	—	—	—	69	—	—	—	71	71	71
8	52·5	—	—	—	—	—	—	—	—	—	—

Broth batch L.2.

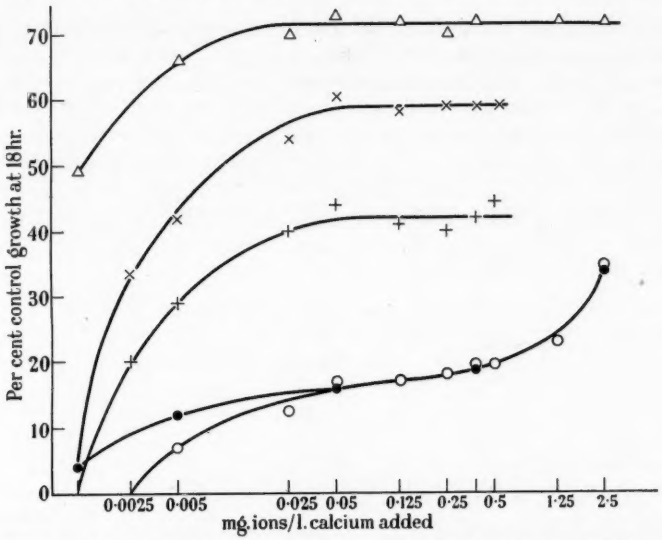


FIG. 3.—Effect on the growth of *Staph. pyogenes* at 18 hr. of the addition of Ca^{2+}, Ca^{2+} and arginine, and Ca^{2+} and Mg^{2+}. (Broth batch L.2.) ○ Ca^{2+}. ● Ca^{2+} and 0·0005 M-arginine. + Ca^{2+} and 0·004 mg. ions/l. Mg^{2+}. × Ca^{2+} and 0·008 mg. ions/l. Mg^{2+}. △ Ca^{2+} and 0·08 mg. ions/l. Mg^{2+}.

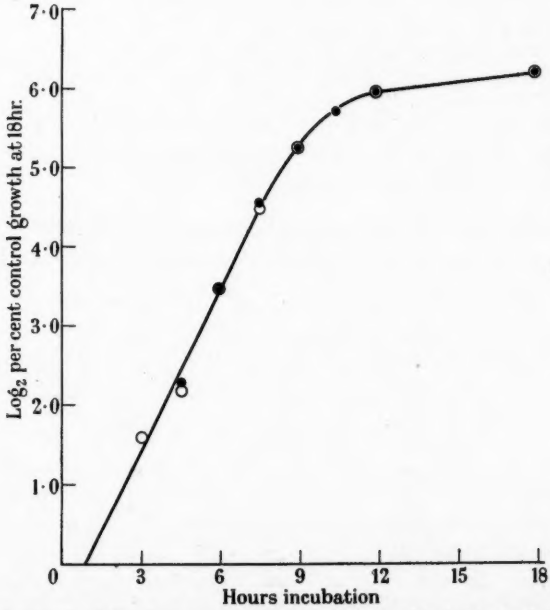


FIG. 4.—Rate of growth of *Staph. pyogenes* in treated broth with 0·04 mg. ions/l. Mg^{2+} and 0·05 mg. ions/l. Ca^{2+}, and 0·8 mg. ions/l. Mg^{2+} and 0·5 mg. ions/l. Ca^{2+}. (Broth batch L.2.) ● 0·04 mg. ions/l. Mg^{2+} and 0·05 mg. ions/l. Ca^{2+}. ○ 0·8 mg. ions/l. $Mg^{2+}$and 0·5 mg. ions/l. Ca^{2+}.

Fig. 4 shows, the rate of growth was the same. In the presence of 0·05 mg. ions/l. Ca^{2+} there was no evidence that Mg^{2+} up to 4 mg. ions/l. depressed growth or that excess Ca^{2+} (up to 2·5 mg. ions/l.) depressed growth in the presence of low concentrations of Mg^{2+} (0·08 mg. ions/l.).

The addition of arginine to the lower concentrations of Mg^{2+} and Ca^{2+} increased growth (Table III), but had no effect at higher levels (Table III and Fig. 2).

TABLE III.—*Effect of the Addition of Arginine to Low Concentrations of Mg^{2+} and Ca^{2+} on the Growth of* Staph. pyogenes *at 18 hr. Results Expressed as a Percentage of Control Growth.*

Magnesium mg.ions/l. added.	Calcium mg.ions/l. added. —	Arginine* only.	0·0025.	0·005	0·005 + arginine.*	0·025.	0·05.	0·05 + arginine*
—	0	4	0	7	12·5	12·5	17	16
0·004	0	20	20	29	35·5	40	44	44
0·008	0	31	33·5	42	51	54	61	60
0·02	11	51	—	59	—	69	71	72

* Arginine = 0·0005 M. Broth batch L.2.

Effect of potassium on growth.

The results of adding K^+ to the experimental medium varied. Potassium alone or K^+ with arginine were ineffective, and the addition of K^+ with Ca^{2+} produced no more growth than would Ca^{2+} by itself (Fig. 5). When K^+ was added with Mg^{2+}, however, growth began with less Mg^{2+} than with Mg^{2+} alone (Table IV) and, as Fig. 5 shows, when 0·1875 mg. ions/l. of K^+ were present with only 0·02 mg. ions/l. Mg^{2+} there was a considerable increase in growth. Larger amounts of K^+ had no greater effect. The increased growth when K^+ was added with Mg^{2+} was mainly evident at sub-optimal Mg^{2+} concentrations, and as may be seen in Table IV ran parallel with the extra growth obtained by the addition of arginine to Mg^{2+}. Arginine, K^+ and Mg^{2+} together produced no better effect than Mg^{2+} with either substance separately.

TABLE IV.—*Effect of the Addition of Arginine and K^+ with Mg^{2+} on the Growth of* Staph. pyogenes *at 18 hr. Results Expressed as a Percentage of Control Growth.*

Magnesium mg.ions/l. added.	—	Arginine.*	Potassium 0·25 mg.ions/l.	Arginine* and 0·25 mg.ions/l. potassium.
0·008	0	31	28	31
0·02	11	51	45	—
0·8	52·5	65	64	—
4	51	54	55·5	—

* Arginine = 0·0005 M. Broth batch L.2.

There was evidence (Fig. 5) that increasing K^+ depressed growth in the presence of low but adequate amounts of Mg^{2+} and Ca^{2+} (0·04 mg. ions/l. and 0·05 mg. ions/l. respectively) to the level reached when Ca^{2+} was absent and arginine present. At higher concentrations growth was increased. By adding 2·5 mg. ions/l. of K^+ to 0·4 mg. ions/l. of Mg^{2+} and 0·5 mg. ions/l. of Ca^{2+} growth could be

increased from 70 to 82 per cent, and the addition of the same amount of K^+ to 4 mg. ions/l. of Mg^{2+} and 2·5 mg. ions/l. of Ca^{2+} increased growth from 71 to 89 per cent.

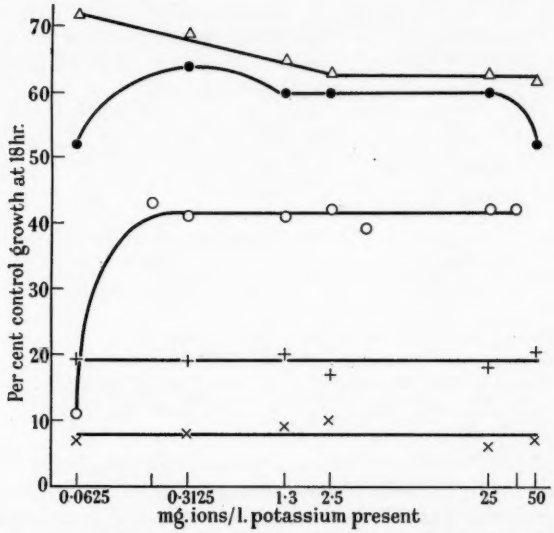


FIG. 5.—Effect on the growth of *Staph. pyogenes* at 18 hr. of the addition of K^+ with Ca^{2+}, and Mg^{2+}. (Broth batch L.2.) × K^+ and 0·005 mg. ions/l. Ca^{2+}. + K^+ and 0·5 mg. ions/l. Ca^{2+}. ○ K^+ and 0·02 mg. ions/l. Mg^{2+}. ● K^+ and 0·8 mg. ions/l. Mg^{2+}. △ K^+ and 0·05 mg. ions/l. Ca^{2+} and 0·04 mg. ions/l. Mg^{2+}.

Effect of the addition of arginine, lysine and sodium.

Arginine.—The addition of arginine at 0·0005 M to the control medium resulted in no increase in growth. Other results with arginine have been mentioned in preceding sections.

Lysine.—Paper chromatography showed lysine to be present in the medium before and after passage through the resin, and lysine did not increase growth when added to Mg^{2+}, Mg^{2+} and arginine, Mg^{2+} Ca^{2+} and arginine.

Sodium.—As the exchange resin was used in the Na^+ cycle, the experimental medium as used contained considerably more Na^+ than the control (approximately 70 mg. ions/l. against 15 mg. ions/l). The addition of 110 mg. ions/l. of Na^+ to the control medium did not depress growth.

General characteristics of growth in deficient media.

There was no gross change in the morphology of staphylococci grown in deficient media as judged by Gram stained preparations. Cultures in treated media with Mg^{2+} only were abnormal in that the bulk of the fluid was almost clear, bacteria being aggregated in specks on the sides and bottom of the tube, although a uniform suspension could be produced by vigorous shaking. Addition of Ca^{2+} resulted in greater uniformity of growth, and cultures, although less dense, appeared otherwise normal.

Growth of other strains of Staph. pyogenes.

The results so far had been obtained with one strain (Terry) of *Staph. pyogenes*. Table V shows the results of an experiment in which the requirements of Terry were compared with those of three other strains. Although there was some variation in the total amount of growth of the four strains at 18 hr., when the results with added Mg^{2+} and Ca^{2+} were expressed as a percentage of the control growth of each strain there was very little difference.

TABLE V.—*Growth at* 18 *hr. of* 4 *Strains of* Staph. pyogenes *with Varying Concentrations of* Mg^{2+} *and* Ca^{2+}. *Results Expressed as a Percentage of the Control Growth of each Strain.*

Magnesium mg.ions/l. added.	Calcium mg.ions/l. added.	Strains of *Staph. pyogenes*.			
		Terry.	Berkof.	Clarke.	Eldridge.
—	0·125	7	8	7	6
0·4	—	20	21	22	25
0·02	0·025	43	43	40	40
		Broth batch L.3.			

Strains Berkof and Clarke were sensitive to penicillin and oxytetracycline. Eldridge was resistant to both antibiotics ; Terry was resistant to penicillin and sensitive to oxytetracycline.

Growth on solid media.

On media consisting of treated broth and agar prepared with HCl, staphylococci either failed to grow or formed a few minute colonies after 18 to 36 hr. Preliminary observations on our solid medium have shown that Mg^{2+} is necessary, and that the effect of K^+ is more noticeable than it is in fluid medium. In particular K^+ is associated with the formation of golden pigment. Deficiencies in the medium resulted in colonies varying in shape, size and depth of pigmentation.

DISCUSSION.

Nicholas (1953) has stated that a mineral element may be shown to be essential for an organism either by removing the metal from the basal culture solution and demonstrating that subsequent graded additions of it result in a specific, statistically quantitative and reproducible increase in growth, or by determining a specific function for it in metabolism. On the first of these grounds our findings with a single strain agree with those of Webb (1951) that magnesium is needed for the growth of *Staph. pyogenes* and also suggest that calcium is required. This may not of course apply to all strains, but the results shown in Table V suggest that the three other staphylococci tested needed magnesium and calcium. These results provide no clue as to the rôle of magnesium, but the interaction of arginine with magnesium and calcium hint at one function for the latter element.

Paper chromatography detected only a trace of free arginine in our treated medium, and as arginine is known to be necessary for the rapid growth of *Staph. pyogenes* (Gladstone, 1937), the excellent growth which the control medium supported was presumably dependent on the breakdown of more complex arginine containing substances. If this is so, an explanation of the results in a deficient medium shown in Fig. 2 may be that the growth level with magnesium only represents growth in an arginine-deficient medium, and that the better level seen when arginine is added to magnesium represents the results of relieving this

deficiency. Haines (1933) showed that proteases were formed in the presence of calcium, and the better level still, seen when 0·05 mg. ions/l. of calcium was added to magnesium, may be due to the presence of sufficient calcium to form proteases, enabling staphylococci to obtain arginine and other amino acids. In this case the addition of arginine to calcium and magnesium would not be expected to stimulate further growth, as in fact it does not. It is possible that potassium can act in a similar way, since, as shown in Table IV, the addition of potassium to magnesium produces almost the same result as the addition of arginine, and adding arginine to magnesium and potassium results in no increased growth. The effect of arginine was not due to the addition of potassium as a contaminant, since no potassium was detected in the arginine by flame photometry.

While removing sufficient ions to create a deficient medium, exchange resins do not remove all of any one ion present, and the results described with calcium and magnesium were obtained in media containing traces of both elements. Other metals were present, and in view of the present lack of knowledge of the requirements of staphylococci for minor elements and the possibility that a deficiency of these elements may have been induced, no explanation is advanced for the reproducible depression of growth found at higher concentrations of magnesium in the presence of arginine or potassium.

The results using solid media have confirmed the findings of Lacey (1954) that the appearance of bacterial colonies can be changed by altering the ionic composition of the medium in which they are grown, and we intend to see if change in appearance of staphylococcal colonies is associated with the change in antigenic composition which Lacey demonstrated for organisms of the *Haemophilus* group. In order to do this, and to determine the needs of *Staph. pyogenes* for the formation of toxins and such substances as hyaluronidase, more information is first wanted of the requirements of this organism for minor elements.

SUMMARY.

In order to determine the mineral requirements for growth of *Staph. pyogenes* a deficient medium has been created by passing nutrient broth through an ion exchange resin. The return of magnesium and calcium to this broth has allowed a reproducible and significant amount of bacterial growth, and it appears that for the strain of *Staph. pyogenes* tested these elements are essential nutrients. The medium used was deficient in free arginine, and the interactions of calcium, magnesium, potassium and arginine suggest that calcium and possibly potassium are required for the formation by this organism of proteases.

On a solid medium considerable variation in colonial appearance could be produced by changing the ionic composition of the medium.

We would like to express our thanks to the Treasurer and Board of Governors of St. Bartholomew's Hospital for a grant which enabled this work to be carried out. To Dr. D. J. D. Nicholas we are indebted for advice in the early stages of the investigation, and throughout to Dr. H. E. Archer.

REFERENCES.

BURTON, MARGARET O., CAMPBELL, J. J. R. AND EAGLES, B. A.—(1948) *Canad. J. Res.*, C, **26**, 15.

GLADSTONE, G. P.—(1937) *Brit. J. exp. Path.*, **18**, 322.

HAINES, R. B.—(1933) *Biochem. J.*, **27**, 466.
HENRY, H. AND STACEY, M.—(1946) *Proc. roy. Soc.*, B, **133**, 391.
KING, JOYCE V., CAMPBELL, J. J. R. AND EAGLES, B. A.—(1948) *Canad. J. Res.*, C, **26**, 514.
LACEY, B. W.—(1954) *Biochem. J.*, **56**, xiv.
NICHOLAS, D. J. D.—(1953) In 'Science and Fruit.' Ed. by Wallace, T. and Marsh, R. W. University of Bristol.
SHANKAR, K. AND BARD, R. C.—(1952) *J. Bact.*, **63**, 279.
WEBB, M.—(1949) *J. gen. Microbiol.*, **3**, 410.—(1951) *Ibid.*, **5**, 485.

THE INFLUENCE OF THE THYROID GLAND ON THE PRODUCTION OF ANTITOXIN IN THE GUINEA-PIG.

D. A. LONG AND JENNIFER SHEWELL.

From the National Institute for Medical Research, Mill Hill, London.

Received for publication March 22, 1955.

PROLONGED treatment with thyroxine increases sensitivity to tuberculin in guinea-pigs (Long and Miles, 1950), whereas a single injection of thyrotrophic hormone, of thyroxine, or of tri-iodo-thyronine is without significant effect (Long, unpublished). Prolonged treatment with thyroxine induces hypertrophy of the islets of Langerhans in rats (Houssay, Foglia and Martinez, 1946) and in guinea-pigs (Long and Shewell, unpublished). Partial pancreatectomy abolishes and insulin restores thyroxine-induced hypersensitivity (Long and Shewell, 1954). It is concluded that thyroxine increases sensitivity to tuberculin in guinea-pigs by inducing hyperinsulinism (Long and Shewell, 1954).

In the present paper, the influence of the thyroid gland on the production of diphtheria antitoxin is considered. There is a close analogy between the effect of the thyroid gland on sensitivity to tuberculin and on synthesis of diphtheria antitoxin. Preliminary experiments showed that treatment of guinea-pigs with thyroxine greatly increased both the level of circulating antitoxin and associated immunity to intradermally injected diphtheria toxin. Indeed, the latter provided a measure of the former in thyrotoxic, as in non-thyrotoxic, guinea-pigs (Hartley, 1934; Miles, 1949; Long, 1950). The increase in immune response was greatest when treatment with thyroxine was prolonged and the dose repeatedly adjusted so that the animals continuously gained a little weight. An association was noted between a high level of circulating antitoxin and hypertrophy of both the adrenal cortex and the islets of Langerhans. Of the two, adrenocortical hypertrophy was considered to be more likely to cause hyperimmunity. Thyrotoxicosis is associated in man with lymphoid hyperplasia. In addition, at the time of these experiments, it had recently been suggested that cortisone induced lympholysis with liberation of antibody (Dougherty and White, 1947).

These ideas were combined in a single hypothesis, that under the influence of the thyroid and adrenocortical hormones together there was a high rate of lymphoid hyperplasia and dissolution with an increased rate of liberation of antibody. A series of experiments completely destroyed this hypothesis by breaking every link in the argument. These proved conclusively that thyroxine-induced hyperimmunity was not mediated through the adrenal cortex, and indeed that in the guinea-pig the activity of the adrenal cortex had little influence on antitoxin production (Long, unpublished). It was therefore decided to investigate the alternative relationship postulated, namely, that between the thyroid gland and the pancreas.

In the present paper, partial pancreatectomy carried out at the end of a course of thyroxine treatment and immediately before the injection of antigen is shown

to prevent increased production of diphtheria antitoxin. It is concluded that thyroxine not only influences sensitivity to tuberculin (bacterial allergy) *viâ* the pancreas, but also antitoxic immunity.

METHODS.

In each of the experiments described, all treated and control groups consisted of 15 albino guinea-pigs (approx : 350 g. weight) of the Hampstead strain, which were assigned at random to each group. Animals were fed upon Bruce and Parkes (1947) Diet 18, hay and unlimited cabbage. Whenever one group was operated upon, all groups, operated or not, received a daily supplement of milk for the rest of the experiment. In these, as in all experiments carried out in this laboratory, only International or Laboratory Standard antigens or antitoxins were used. By such means results obtained in different experiments can be compared, and the doses of immunising antigen and test toxin likely to yield maximal accuracy employed.

The immunising antigen was a Laboratory Standard alum-precipitated diphtheria toxoid (Ba 536). Under the conditions of these experiments, 0·5 LF in 1 ml. of physiological saline injected into the adductor muscles of the right hind leg, followed, at an interval of not less than 28 days, by an injection into the same site of a second similar dose of antigen, produces, after a further interval of 10 days, a sub-maximal antitoxin response falling on the steepest part of the curve. In Experiments I and II, the first dose of antigen (the primary stimulus) was given 4 weeks, and in Experiment III it was given 15 weeks, before the second dose of antigen (the secondary stimulus).

Test toxin T.P. 2776, a Laboratory Standard that has maintained constant potency (as judged by LR/10,000 and M.R.D. estimations) since 1948, was used for all multiple Schick tests and for direct assay of antitoxin. The International Standard for Diphtheria Antitoxin and, in addition, a Laboratory Standard for guinea-pig antitoxin (to avoid confusion over problems of differing avidity) were used to determine potency of sera. In all cases the titres of sera were assayed against both standards by two methods—by a modification of the classic Römer and Sames technique (1909) and Miles' (1949 ; 1954) indirect neutralisation technique. Miles (1949) showed that the response obtained when the inflammatory lesion diameter at 24 hr. is plotted against the log. dose of toxin in passively immunised guinea-pigs is linear ; different dose-response curves are substantially parallel, and the shift in the dose-response curve is proportional to the antitoxin content of the blood. By fitting regression lines to the dose-response curves so obtained, it is possible to detect relatively small differences in the antitoxic immunity in different groups of animals ; by analysis of variance, the errors of the test and the significance of differences in the various experimental groups can be accurately determined.

Further experiments (Long, 1950 ; Miles, 1954) showed that the same relations held in actively immunised guinea-pigs (cf. Hartley, 1934), so that substantially the same method was used for measuring active immunity and immunity passively conveyed with serum to previously non-immune guinea-pigs. The latter provides a true measure of circulating antitoxin for neutralisation of injected toxin is in this case independent of hormonal effects. In practice, results obtained in actively or passively immunised animals were identical.

Thus, in all experiments, the immune response was measured by the intradermal injection of graded doses of diphtheria toxin, using this modification of the classic multiple Schick technique.

Experiment I.

Two groups of 15 guinea-pigs were used. Both groups were given a first dose of antigen 4 weeks before the secondary stimulus. Two weeks before the secondary stimulus one group was treated with thyroxine. Sodium thyroxine solution, 0·20 mg./kg. body wt. was injected subcutaneously three times a week for 2 weeks, so that at the time of the secondary stimulus a severe thyrotoxicosis of 2 weeks' duration had been produced.

Experiment II.

To study the effect of prolonged mild thyrotoxicosis on antitoxin formation one group of 15 guinea-pigs was injected subcutaneously with sodium thyroxine solution, 0·10 mg./kg. body wt., twice weekly for 18 weeks. A second group of animals was given 50 mg./kg. body wt. propylthiouracil orally three times a week for the same period. After 14 weeks'

treatment these and two untreated groups were given a first dose of antigen, followed 4 weeks later by the secondary stimulus.

Experiment III.

To study the effects of partial pancreatectomy and thyrotoxicosis on antitoxin production in the guinea-pig, 4 groups of 15 animals were given a first dose of antigen. After 5 weeks two groups were given 0·10 mg./kg. body wt. sodium thyroxine solution subcutaneously once a week for 10 weeks. Five to 8 days before the secondary stimulus was given, 15 weeks after the first stimulus, the guinea-pigs from one of the thyroxine-treated groups and from one of the untreated groups were partially pancreatectomised under ether anaesthesia. The other two groups of animals were sham-operated under ether anaesthesia at the same time. Approximately $\frac{2}{3}$ of the pancreas was removed, the head of the pancreas being left intact in order to avoid damaging the pancreatic ducts. Post-mortem examination showed that in each case an adequate amount of functional pancreas remained.

Electrophoretic analysis of sera in each experiment failed to show a significant difference between groups.

RESULTS.

Experiment I.

Severe thyrotoxicosis of 2 weeks' duration caused a significant ($P < 0·001$) 2·4-fold increase in immunity to intradermally injected diphtheria toxin. This was associated with a corresponding increase in circulating antitoxin.

Experiment II.

Mild thyrotoxicosis of 18 weeks' duration caused a significant ($P < 0·001$) 12·8-fold increase in immunity to intradermally injected diphtheria toxin (Table I).

TABLE I.—*Effect of Mild Thyrotoxicosis of* 18 *Weeks' Duration on the Secondary Response to Diphtheria Toxoid. Comparison of Individual Treatment Effects with the Effect in Controls.*

Treatment.	Dose.	Secondary response.	F.	P.
Sodium thyroxine	0·1 mg. twice weekly for 18 weeks	12·8-fold increase	16·5	<0·001
Propylthiouracil	50 mg./kg. thrice weekly for 18 weeks	1·5-fold decrease	<1	—

Comparison of antitoxin values obtained by different techniques.

	Modified multiple Schick method (Long, 1950) (u./ml.).	Indirect neutralisation method (Miles, 1949) (u./ml.).	Römer and Sames method (1909) (u./ml.).
Nil	4	4	4
Sodium thyroxine	71·2	60	70

F = ratio of variance due to the difference between treatments to animal variance. P = probability that the corresponding value of F could occur by chance.

Degrees of freedom.—Sodium thyroxine 1,27. Propylthiouracil 1,27.

This was associated with a corresponding increase in circulating antitoxin. Treatment with propylthiouracil for 18 weeks decreased the immune response, but the effect was not significant. This was associated with a corresponding decrease in circulating antitoxin. Similar experiments have consistently shown a decrease in immunity of approximately 1·5-fold, but with a group of this size animal variation has always been too great for the difference to be shown to be

significant. The effect is probably real; propylthiouracil tends, if anything, to depress antitoxin synthesis in guinea-pigs.

Experiment III.

Very mild thyrotoxicosis (B) of 10 weeks' duration caused a significant increase in immunity to intradermally injected diphtheria toxin (Table II). Partial pancreatectomy (A) caused a slight but not significant decrease in immunity. Partial pancreatectomy carried out in thyrotoxic guinea-pigs (A + B) immediately before the second injection of antigen (the secondary stimulus) caused a slight but not significant decrease in immunity to intradermally injected diphtheria toxin. In these, as in all experiments quoted, immunity to diphtheria toxin was associated with comparable changes in the level of circulating antitoxin.

TABLE II.—*Effect on the Secondary Response to Diphtheria Toxoid of Very Mild Thyrotoxicosis of 10 Weeks' Duration in Partially Pancreatectomised, and in Intact Guinea-pigs. Comparison of the Individual Treatment Effects with the Effect in Controls.*

Treatment.	Potency ratio.	F.	P.
A	1·35 (0·74-fold)	<1	—
B	0·41 (2·5-fold)	6·73	0·05–0·01
A + B	1·21 (0·83-fold)	<1	—

	High dose toxin.		Low dose toxin.	
	Mean.	Range.	Mean.	Range.
Controls	24·4	(20·5–27·0)	18·1	(15·0–21·0)
A	25·4	(17·5–32·0)	19·1	(13·0–24·25)
B	21·9	(15·0–27·0)	15·9	(7·0–20·0)
A + B	25·0	(15·0–30·0)	18·5	(7·5–23·0)

The responses in each treatment group were compared with the control in order to assess the significance of the effect of treatment on immunity.

For example, analysis of variance of Group B *v.* controls.

Source of variation.	Sum of squares.	D.F.	Mean square.	F.	P.
Between treatments	82·2510	1	82·2510	6·73	0·05–0·01
Regression	562·7344	1	562·7344	313·22	<0·001
Departure from parallelism	0·0844	1	0·0844	<1	—
Between animals	342·1542	28	12·2198	—	—
Residual	46·7125	26*	1·7966	—	—
Total	1033·9365	57*	—	—	—

Potency = 0·41465. * 1 dead control animal.

Comparison of antitoxin values obtained by different techniques.

Treatment.	Modified multiple Schick (Long, 1950) (u./ml.).	Indirect neutralisation method (Miles, 1949) (u./ml.).	Römer and Sames method (1909) (u./ml.).
—	10	10	10
A	7	>10	>10
B	25	30	40
A + B	8	>10	>10

A = Partial pancreatectomy. B = Sodium thyroxine.

F = ratio of variance due to the difference between treatments to the animal variance, and has (1,28) degrees of freedom. P = probability that the corresponding value of F could occur by chance.

DISCUSSION.

Since the criterion employed in the measurement of antibody is the neutralisation of toxicity, the difference in antitoxin levels cannot be attributed to a modification of the serological (as distinct from the immunological) properties of circulating antibody as a result of hormonal activity. It reflects a difference in immunity.

Thyroxine increases active immunity to intradermally injected diphtheria toxin and this is associated with a comparable increase in circulating antitoxin. The effect is increased if thyroxine is given in a manner calculated to stimulate but not to overwhelm the compensatory endocrinological response.

Partial pancreatectomy carried out towards the end of a course of treatment with thyroxine, and immediately before antigenic stimulation, prevented this increase in immunity. From this it is concluded that treatment with thyroxine causes an increase in immunity *viâ* the pancreas. Additional confirmatory evidence for this conclusion was obtained by a statistical comparison of groups A and B (thyroxine treatment and partial pancreatectomy) with group A (partial pancreatectomy), which showed that thyroxine had no effect in pancreatectomised animals.

The conclusion reached is that treatment of guinea-pigs with thyroxine increases the production of diphtheria antitoxin and that this effect is produced *viâ* the pancreas, presumably *viâ* the islets of Langerhans.

The influence of thyroid activity on antitoxic immunity in guinea-pigs is analogous to its influence on sensitivity to tuberculin. If these two immunological phenomena are representative of allergic and non-allergic responses to bacterial infection it can be claimed that thyroxine, by its effect on the pancreas, increases bacterial allergy and antitoxic immunity.

The importance of species difference in studies of this kind cannot be too strongly emphasised (Perla and Marmoston, 1941 ; Long, 1954). There is a reasonable chance that observations made in the guinea-pig will apply to man (Long, 1954).

SUMMARY.

Using both the response to diphtheria toxin injected intradermally and the assay of circulating antitoxin in guinea-pigs immunised with diphtheria toxoid, it was found that thyroxine increases immunity and that partial pancreatectomy prevents the action of thyroxine in increasing immunity.

It is concluded that thyroxine increases immunity in guinea-pigs by inducing compensatory hypertrophy in the pancreas, presumably of the islets of Langerhans.

We are indebted to Miss M. V. Mussett, B.Sc. for the statistical analyses. The immunological methods employed are based largely on the advice and then unpublished work of Professor A. A. Miles, to whom we acknowledge our indebtedness.

REFERENCES.

Bruce, H. M. and Parkes, A. S.—(1947) *J. Hyg., Camb.*, **45**, 70.
Dougherty, T. F. and White, A.—(1947) *J. Lab. clin. Med.*, **32**, 584.

HARTLEY, P.—(1934) *Wiss. Woche Frankfurt a.M.*, **3**, 81.
HOUSSAY, B. A., FOGLIA, V. G. AND MARTINEZ, C.—(1946) *Endocrinology*, **39**, 361.
LONG, D. A.—(1950) *Brit. J. exp. Path.*, **31**, 183.—(1954) *Lancet*, i, 529.
Idem AND MILES, A. A.—(1950) *Ibid.*, i, 492.
Idem AND SHEWELL, JENNIFER—(1954) *Brit. J. exp. Path.*, **35**, 503.
MILES, A. A.—(1949) *Ibid.*, **30**, 319.—(1954) *Fed. Proc.*, **13**, 799.
PERLA, D. AND MARMOSTON, J.—(1941) 'Natural Resistance and Clinical Medicine.' Boston (Little, Brown & Co.).
RÖMER, P. H. AND SAMES, TH.—(1909) *Z. ImmunForsch.*, **3**, 344.

ANTIBODY FORMATION IN THE OMENTUM.

K. B. ROBERTS.*

From the Sir William Dunn School of Pathology, Oxford.

Received for publication March 29, 1955.

WITH the possible exception of the liver, tissues of the body containing a high proportion of reticulo-endothelial elements are able to produce antibody. The spleen is probably the main site of both the fixation of antigen and the production of antibody when moderate amounts of antigen are given intravenously, while the draining lymph nodes may be more important when antigen is given into the skin (Roberts, Adams and White, 1949 ; McMaster and Hudack, 1935). When particulate material is injected into the peritoneal cavity, it is removed partly by the draining lymphatics and partly by the macrophages of the omental " milk-spots ". Little is known of the rôle of the omentum in antibody formation after intraperitoneal injection. The present work describes the reaction of the omentum of the rabbit to intraperitoneal injections of formalin-killed *Salmonella typhi* organisms (H antigen).

EXPERIMENTAL METHODS.

Young adult rabbits of both sexes weighing from 1·5 to 2·5 kg. were used. The preparation of the antigen and the methods for estimating agglutinin titres were those described in a previous paper (Roberts, 1955). Intraperitoneal injections were made in the anaesthetised animal, the organisms for injection being suspended in up to 2 ml. of 0·9 per cent NaCl.

Estimation of tissue titres.—The animals were killed by exsanguination. Weighed portions of tissues were extracted into distilled water, either by grinding with sand or by homogenising in an ice-cold Potter tube for 1 min. The efficiency of extraction by these two methods was found to be similar ; a sample of omentum and another of spleen, treated in these two ways, gave consistent antibody titres. The extracted tissue was centrifuged and the antibody titre of the supernatant fluid assayed in the usual way. The method of expressing the tissue titres may best be shown by an example. Wt. of tissue : 3·7 g. : 6·3 ml. distilled water added and tissue homogenised. Titre of supernatant, 640. The tissue was " diluted " approximately 1/2·7 ; tissue titre is said to be 640 × 2·7 or 1728, which is given as 1720.

Tissue transfer methods.—Donor rabbits, previously immunised by intraperitoneal injection of *Salm. typhi*, H antigen, were killed by exsanguination. The omentum was removed and finely minced with sharp-pointed scissors. After a small quantity had been taken for antibody assay, the minced tissue was weighed and divided into two portions. One portion was placed in the peritoneal cavity of one of a pair of recipient rabbits through a small mid-line incision. The second portion of tissue was heated in a water-bath at 56° for 30 min. It was then inserted into the peritoneal cavity of the other recipient animal. In these experiments, the recipient pairs were albino, virgin female litter-mates of the same body weight.

Histological techniques.—Tissues were fixed in formol-saline and stained with haematoxylin and eosin. The omentum was also stained by the Unna-Pappenheim method, the section being taken to distilled water, left in methyl-green-pyronine (Carleton, 1938) for 20

* Philip Walker Student, University of Oxford.

min., washed and then differentiated in equal parts of acetone and distilled water for a few seconds.

RESULTS.

Tissue titres of organs after intraperitoneal injection of antigen.

Killed *Salm. typhi* organisms were injected intraperitoneally into 7 rabbits on one or more occasions to give various serum titres of agglutinating antibody. The tissue titres were estimated and were compared with those from 3 other rabbits receiving antigen intravenously. The results are given in Table I.

TABLE I.—*Serum and Tissue Antibody Titres after Immunisation with Killed* Salm. typhi.

Rabbit No.	Number of organisms injected.	Days after last injection.	Serum titre	Tissue titres.				
				Liver.	Spleen.	Node.*	Omentum.	Kidney.
Intraperitoneal route—								
1	10^{11}	5	20	0	0	0	6	0
2	10^{10}	7	640	20	80	—	140	—
3	(2×10^{9}) twice	13	1,280	80	160	60	240	80
4	10^{10}	36	5,120	260	—	460	1,600	480
5	(2×10^{11}) twice	5	5,120	1,200	1,760	680	3,200	—
6	(2×10^{11}) thrice	5	40,960	960	1,200	1,600	3,560	—
7	10^{10} and, 200 days later, 2×10^{11}	8	81,920	960	1,040	1,600	3,360	—
Intravenous route—								
8	10^{11}	10	1,280	120	480	100	100	—
9	2×10^{10}	10	1,280	160	400	120	160	—
10	10^{11}	16	5,120	460	640	220	240	480

* Mesenteric lymph node.

These experiments show that a relatively high level of antibody may be found in the omentum after intraperitoneal, but not after intravenous, injections. The tissue titres of this organ are higher than those of the kidney, liver and lymph nodes, even some weeks after a single intraperitoneal injection when the acute inflammatory response may be assumed to have subsided (Rabbit No. 4).

In a series of 3 rabbits the technique first devised by McMaster and Hudack (1935) was used. An intraperitoneal injection containing 10^{10} killed *Salmonella paratyphi B* was given to a rabbit and, at the same time, 10^{10} killed *Salm. typhi*

TABLE II.—*Serum and Tissue Antibody Titres after Intraperitoneal Injection of Killed* Salm. paratyphi B, *and Simultaneous Injection of Killed* Salm. typhi *Intravenously.*

Rabbit No.		Serum titres.	Tissue titres.			
			Liver.	Spleen.	Node.	Omentum.
1	Against *Salm. paratyphi*	320	40	20	20	80
	„ *Salm. typhi*	640	140	400	60	80
2	Against *Salm. paratyphi*	640	40	60	—	180
	„ *Salm. typhi*	1,280	260	400	—	100
3	Against *Salm. paratyphi*	320	40	20	—	60
	„ *Salm. typhi*	320	60	120	—	40

organisms were given into the ear vein. The animal was killed 10 days later and the serum and tissue agglutination titres against the two antigens were estimated. These are shown in Table II. It can be seen that in each case the omentum has a higher tissue titre than the other organs to the intraperitoneally injected *Salm. paratyphi B* but a relatively low tissue titre to the intravenously injected *Salm. typhi*. This distribution is consistent with the suggestion that the omentum is itself involved in the production of antibody after an intraperitoneal injection of antigen.

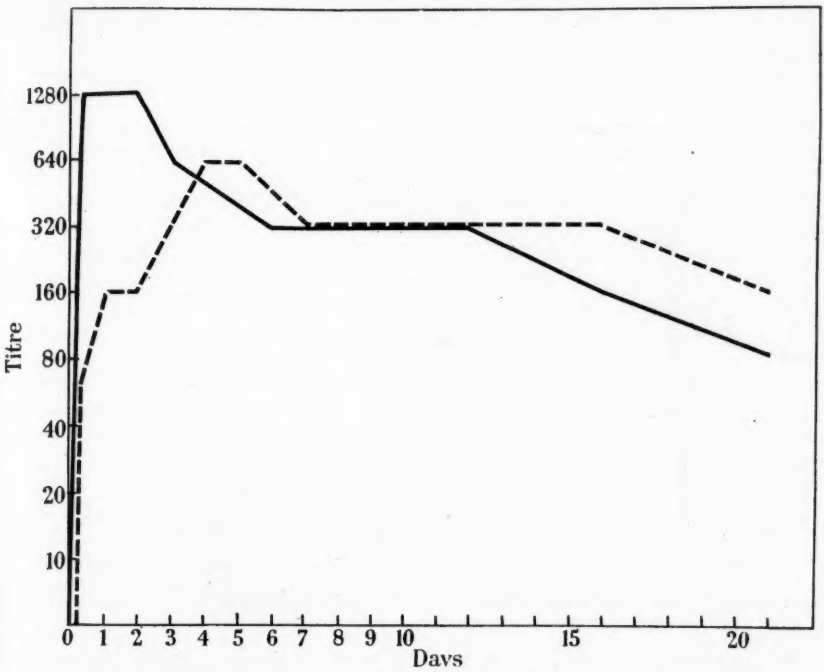


FIG. 1.—The serum agglutinin response in two recipient rabbits. — — — — — Received the donor's omentum. ———— Received the donor's serum.

Omental transfer experiments.

A rabbit was given 10^{11} organisms on alternate days on three occasions. It was killed 5 days after the last injection of antigen when the serum titre was 40,960. The omentum was removed, minced with scissors and 11·5 g. transplanted into the peritoneal cavity of a normal rabbit. Antibodies appeared in the serum of the recipient rabbit within 6 hr. and followed the course shown in Fig. 1. It may be compared with the antibody response, also shown in Fig. 1, of another normal rabbit which had received 11·5 ml. of the donor's serum with a titre of 40,960. The serum titres of these two animals are of the same order even though the 11·5 g. of transferred omentum was estimated to have a tissue titre of only 3360. This

could be explained by new antibody formation in the transplanted omentum, a transfer of antigen to give an active immune response in the recipient, an inefficient method for the extraction of antibody from the tissue, or by a combination of these factors. To overcome this difficulty the following experiment was done. Two intraperitoneal injections of 10^{11} killed organisms were given on consecutive days to a rabbit previously immunised 205 days before with 10^{10} organisms intraperitoneally. Eight days after the last injection, when the serum titre was 81,920, the animal was killed and the omentum was removed, minced and separated into two parts, each weighing 12·6 g. One part was transferred immediately, as described above, into the peritoneal cavity of one of a pair of recipient animals. The other

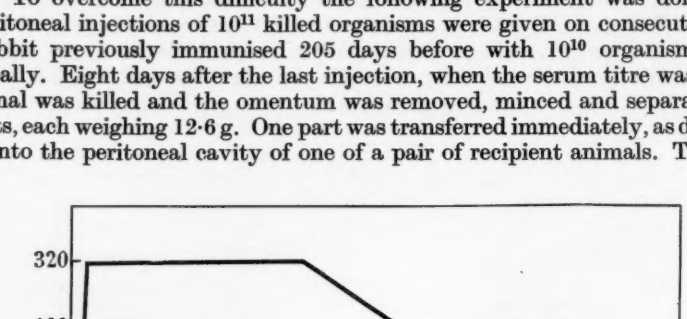


FIG. 2.—The serum agglutinin response in two recipient rabbits. ———— Received living omentum. — — — — — — Received heat-killed omentum.

part was heated to 56° for 30 min. before it was transferred to the second recipient animal. This treatment kills all the cells of the omentum without damaging any antigen or antibody that might be present in the tissue. The serum agglutinin responses in the two recipient animals are shown in Fig. 2. It is probable that the transferred living omentum continued for a while to produce antibody in the peritoneal cavity of the host for it may be seen that there was more antibody at all stages in the serum of the animal receiving living omentum. Two further experiments using this technique yielded similar results.

It may be of interest to note that the transferred omentum in each case contained large numbers of typical plasma cells. In one case the recipient animal was killed 8 days after transfer and the omental pieces examined histologically. The subserosal tissues contained large numbers of plasma cells, apparently normal in structure and in staining reactions, while all other cells had become necrotic. It is impossible to decide the origin of these plasma cells.

Histological observations.

Normal histology.—The structure of the omentum has been given by Portis (1924) and by Maximow (1928). The non-fatty parts of the omentum in the rabbit contain collections of reticulo-endothelial cells ; these are known as " milk-spots ". Elsewhere, except along the vessels, the tissue is relatively acellular.

The omentum from each of 6 normal rabbits was examined after staining with haematoxylin and eosin, and with methyl-green-pyronine. In 4 of these collections of lymphocytes were seen in the vicinity of the milk-spots. A small number of pyronine-staining plasma cells were also seen, but they did not form any considerable proportion of the " round cells " of the organ.

Immunised rabbits.—The changes in the omentum that follow the injection of organisms into the peritoneal cavity have been summarised by Wilson and Miles (1946). They were confirmed in this present work.

Seven rabbits were immunised intraperitoneally with 10^{10} organisms. The animals were killed 6 hr., 1, 5, 9, 13, 36 and 70 days after this injection. The omentum of those killed at 6 and 24 hr. showed a predominantly polymorphonuclear response which was followed later by one composed largely of macrophages. It was noticed, however, that at 5 days and subsequently there was, superimposed on this inflammatory response, a new formation of pyronine-staining cells. These appeared at first in the thickened subserosal tissues, especially in the hypertrophied milk-spots. A similar reaction was seen in 2 rabbits receiving 10^{11} organisms intraperitoneally, repeated on two and on three occasions, the last injection being given 6 and 5 days respectively before the animal was killed. In these animals, pyronine-staining cells had infiltrated throughout the greatly hypertrophied and fibrotic omentum. Cells similar to those described by Fagraeus (1948) as transitional cells, immature plasma cells and typical plasma cells were all present, but the majority of the pyronine-staining cells were of the last-named variety. The cells of lymphoid foci present in the omenta did not stain with pyronine. In the second of these two animals it could be seen that some portions of the omentum were composed entirely of plasma cells embedded in loose connective tissue.

The omentum from a rabbit immunised intravenously was similar in structure to that from a normal animal.

DISCUSSION AND CONCLUSIONS.

Portis (1924), among others, has shown that antigenic particles are taken up, when injected into the peritoneal cavity, by the macrophages of the omental milk-spots. The experiments reported here show that a relatively high level of antibody may be found in the omentum after intraperitoneal, but not after intravenous injection. From the experiments involving transfer of heat-killed omentum, it is probable that the tissue titre is much higher than that estimated from extraction *in vitro*. Experiments in which an injection of one antigen was made into the peritoneal cavity while another, but similar antigen was given intravenously, again suggest that antibody is being formed in the omentum and not merely localised in inflamed tissues.

The transfer experiments indicate that the omentum may continue to produce antibodies in the peritoneal cavity of another rabbit. The homograft cannot, of course, be expected to survive in this environment for more than a few days.

There is a marked plasma cell response in the omentum after an intraperitoneal

injection of antigen. This finding is in agreement with those of many others correlating the formation of plasma cells with antibody production (Fagraeus, 1948).

The work of Portis (1924) also suggests that the omentum may be a site of antibody formation, for he found that surgical removal of the omentum in rabbits lowered the immune response to intraperitoneal but not to intravenous injections of sheep erythrocytes.

SUMMARY.

When formalin-killed *Salm. typhi* organisms were injected into the peritoneal cavity of rabbits there was a higher level of antibody in the omentum than in the spleen, liver, kidney or mesenteric lymph node.

Injections of killed *Salm. paratyphi B* were made into the peritoneal cavity, while killed *Salm. typhi* were given intravenously. The resulting tissue antibody titres indicated an active formation of antibody by the omentum.

There is some indication that living omental tissue from rabbits which have been immunised intraperitoneally can continue to form antibody when transferred into the peritoneal cavity of normal recipient rabbits.

After an intraperitoneal injection of killed *Salm. typhi* organisms, there is an acute inflammatory response in the omentum. Superimposed on this is a new formation of pyronine-staining cells, many of which are typical plasma cells.

The author wishes to thank Professor Sir Howard Florey for his advice and encouragement. My thanks are also due to Mr. H. Axtell, who prepared the histological sections, and to Miss C. Court, who drew the graphs.

REFERENCES.

CARLETON, H. M.—(1938) 'Histological Technique.' 2nd ed. London (Oxford Medical Publications).
DARCY, D. A.—(1949) *Nature, Lond.*, **163**, 98.
FAGRAEUS, A.—(1948) *Acta med. scand.*, Suppl., 204.
MCMASTER, P. D. AND HUDACK, S. S.—(1935) *J. exp. Med.*, **61**, 783.
MAXIMOW, A. A.—(1928) In Cowdry's 'Special Cytology.' New York (Hoebber).
PORTIS, B.—(1924) *J. infect. Dis.*, **34**, 159.
ROBERTS, K. B.—(1955) *Brit. J. exp. Path.*, **36**, 199.
ROBERTS, S., ADAMS, E. AND WHITE, A.—(1949) *J. Immunol.*, **62**, 155.
WILSON, G. S. AND MILES, A. A.—(1946) Topley and Wilson's 'Principles of Bacteriology and Immunity.' 3rd ed. London (Arnold), p. 1040.

THE EFFECT OF CULTURAL CONDITIONS ON THE SUSCEPTIBILITY OF *BACILLUS ANTHRACIS* TO LYSOZYME.

G. P. GLADSTONE AND H. H. JOHNSTON.

From the Sir William Dunn School of Pathology, University of Oxford.

Received for publication April 1, 1955.

IN contrast to *Bacillus megatherium* there is general agreement that *Bacillus anthracis* is resistant to the action of lysozyme (Amano, Kato and Shima, 1953; Tomcsik, 1954; Herbert, personal communication, 1945). Some early reports suggesting that it was susceptible (Wolff, 1927; Melnik, 1936) were of work carried out before lysozyme had been prepared in a relatively pure crystalline state by Abraham and Robinson (1937). There is no evidence that the antibacterial action of the material used in this work, such as crude egg white and extracts of animal tissues, was due to lysozyme.

In the course of a study of the effects of serum and extracts of leucocytes and tissues of normal rabbits on *B. anthracis*, tests with lysozyme were included more to act as negative controls than in the expectation that there would be any bactericidal action. All strains of *B. anthracis* when grown in air were, in fact, completely resistant to a concentration of lysozyme as high as one per cent. However, certain strains were found to be susceptible when grown with 0·05–0·1 M sodium bicarbonate in 10–20 per cent CO_2. Investigations of the action of lysozyme on these strains and of the conditions determining their sensitivity form the subject of this paper.

TECHNICAL DETAILS.

Strains of B. anthracis.

These were as follows: 109, 1607, 1712, 2620 and 5444, from the National Collection of Type Cultures, Colindale; "Sterne", the non-capsulated variant used for the manufacture of living spore vaccine supplied by Dr. M. Sterne of the Wellcome Research Laboratories, Langley Court, Beckenham, Kent; N.P.A., the virulent non-proteolytic mutant derived from "Vollum" after exposure to ultraviolet light (Wright, Hedberg and Feinberg, 1951); M 36, a virulent strain received from Dr. D. W. Henderson of the Microbiological Research Department, Ministry of Supply, Porton, Wilts. From this last strain a number of colonial variants were selected from growth on a CCY (Gladstone and Fildes, 1940) agar plate. These were designated A, B, B/R, C, S and V. These, with the exception of B/R, which was a rough non-capsulated variant selected by culturing B on plates in 20 per cent CO_2 (Sterne, 1937), were all typical capsulated strains of *B. anthracis*.

Spore suspensions.

Unless otherwise stated spore suspensions were used as culture inocula and for virulence tests. These were prepared by culturing the organisms on CCY agar slopes in 8 oz. (215 ml.) medical flats. The 48-hr. growth was washed off, centrifuged and re-suspended in glass-distilled water, heated at 70° for 30 min. and standardised by viable count (Miles and Misra, 1938) to contain approximately 10^8 spores per ml. No appreciable fall in count occurred after storage at 4° for one year.

Basal culture medium.

This was a modification of CCY broth. It was made up as follows.

Bacto-Vitamin Free Casamino Acids 20 g.; *dl* tryptophan 0·324 g.; 70 per cent Na lactate 142 ml.; Na glycerophosphate 20 g.; $MgSO_4$ 0·02 g.; $MnSO_4$ 0·008 g.; iron solution (see below) 10 ml.; yeast extract (Gladstone and Fildes, 1940) 200 ml.; glass-distilled water to 1,000 ml.

(The iron solution was made by dissolving ferrous ammonium sulphate 0·0128 g. and citric acid 0·0128 g. in 100 ml. distilled water.) The pH was adjusted to 7·6 electrometrically. The medium was then dispensed in 100 ml. volumes in screw-capped bottles, sterilised by autoclaving at 15 lb. per sq. in. (1055 g. per sq. cm.) for 15 min. and stored at 4°.

For use, the medium was diluted 1/2 with sterile glass-distilled water, allowance being made for any further additions as mentioned under "Experimental". For surface cultures the medium was solidified with 2 per cent Bacto-agar.

Culture vessels.

These were Pyrex 6 × $\frac{5}{8}$ in. (15·2 × 1·6 cm.) test tubes bent at right angles half way along their length. Each contained 2 ml. of medium added aseptically.

Inoculum.

Unless otherwise stated, 1000 spores were inoculated in 2 ml. of medium.

Incubation.

Tubes were shaken continuously in the plane of the long axis of the bent tube (50 shakes per min., amplitude of shake 5 cm.) in a water bath at 37°. Tubes to be incubated in various concentrations of CO_2 were partially evacuated, refilled to atmospheric pressure with pure CO_2 and sealed with rubber bungs. In such tests uninoculated tubes were included as a control for pH.

Estimation of growth.

This was made by measurements on an EEL photoelectric colorimeter.

Viable counts.

Serial dilutions were made with graduated dropping pipettes in 1 per cent Bacto-Vitamin Free Casamino Acids containing 0·02 M phosphate buffer at pH 7·6. The technique of counting was that of Miles and Misra.

Sodium bicarbonate.

A molar solution of $NaHCO_3$ (B.D.H. "Analar" reagent standard) was prepared in neutral glass-distilled water and sterilised by Seitz-filtration under positive pressure. The solution was stored at 4° in sealed bottles. It was added aseptically to the medium.

Lysozyme.

Most experiments were carried out with crystalline lysozyme prepared from egg white by Armour Laboratories. A highly purified (six times recrystallised) sample supplied by Dr. G. Alderton was also used. Stock solutions 1/100 w/v in distilled water were sterilised by Seitz-filtration and stored at 4°.

EXPERIMENTAL.

Sensitivity to Lysozyme on Gutter-plates Incubated in Air.

A survey of the sensitivity of *B. anthracis* strains to lysozyme in air was made by streaking each strain across plates containing lysozyme in a gutter.

Fig. 4 shows that all strains were completely resistant to a concentration of lysozyme of 1/2000.

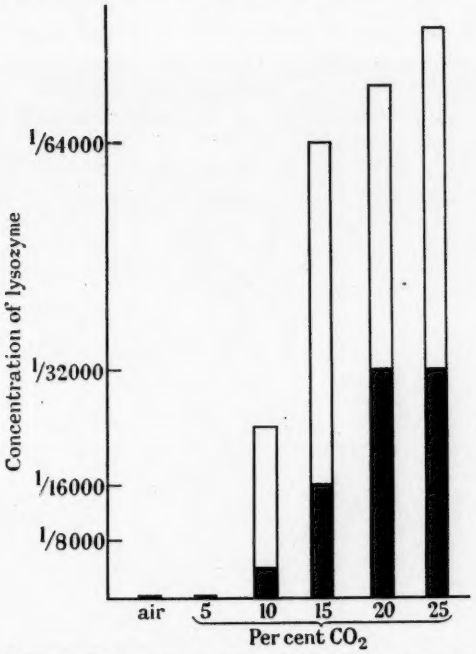


FIG. 1.—Inhibition of growth of *B. anthracis* strain B at various concentrations of CO_2. In each case the pH is held at 7·5 by use of the appropriate concentration of $NaHCO_3$. Complete inhibition of growth ■; 50 per cent inhibition of growth □.

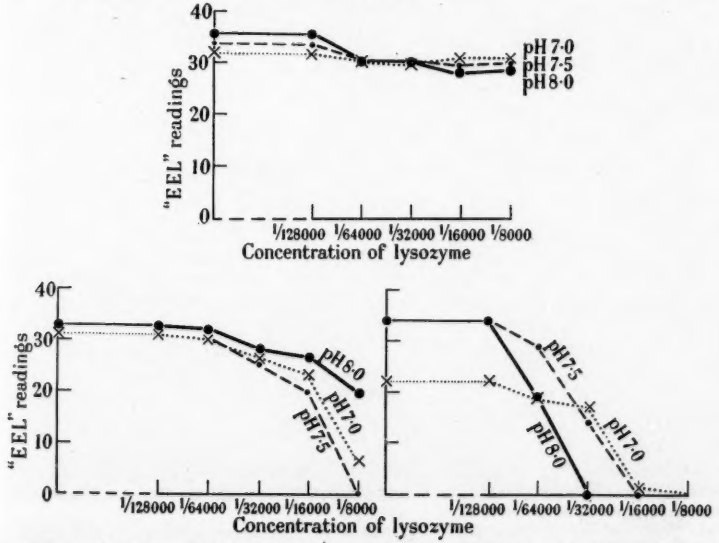


FIG. 2.—Effect of bicarbonate on titrations of lysozyme with *B. anthracis* strain B. The three pH values used with each concentration of HCO_3 were achieved by varying the CO_2 concentration in which the cultures were grown.

In FIG. 2 of the paper by G. P. Gladstone and H. H. Johnston, p. 365, the 'HCO_3 concentrations for the graphs were omitted. They were :—

upper graph	0·025 M
lower left graph	0·05 M
lower right graph	0·1 M

Sensitivity to Lysozyme on Gutter-plates Incubated in 20 per cent CO_2.

Similar tests were carried out in 20 per cent CO_2, 0·1 M sodium bicarbonate being incorporated in the medium to maintain the pH at 7·6. Although most strains were still resistant, certain of the colonial variants of the M 36 strain (B, B/R, D and S) were now found to be susceptible (Fig. 5). N.C.T.C. 1712 (Pasteur's " Deuxième Vaccin " strain) was also found to be inhibited, but to a lesser extent than the M 36 variants. Two of these variants, A, unaffected by lysozyme, and B, lysozyme susceptible, were chosen for further study.

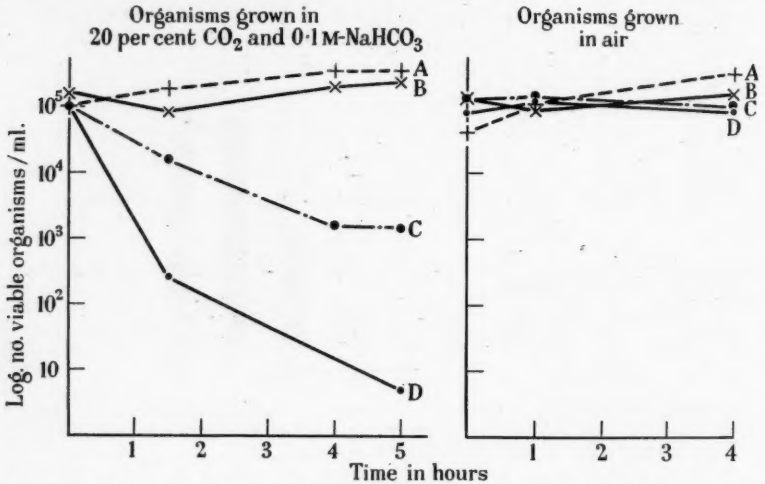


FIG. 3.—Effect of lysozyme on the viable count of *B. anthracis* strain B grown in the presence and absence of 0·1 M-$NaHCO_3$ and 20 per cent CO_2.

Test conditions: A: No $NaHCO_3$; no lysozyme. B: With 0·1 M-$NaHCO_3$; no lysozyme. C: No $NaHCO_3$; with 1/2000 lysozyme. D: With 0·1 M-$NaHCO_3$ and 1/2000 lysozyme.

The Quantitative Estimation of Lysozyme Sensitivity.

When lysozyme was added to a liquid culture of B grown in 20 per cent CO_2 with 0·1 M-$NaHCO_3$ in the medium, no decrease in opacity was observed but, on the contrary, the suspension greatly increased in opacity. This phenomenon was demonstrated by all capsulated strains of *B. anthracis* irrespective of whether they were sensitive to lysozyme or not. It is due to a reaction between the poly-

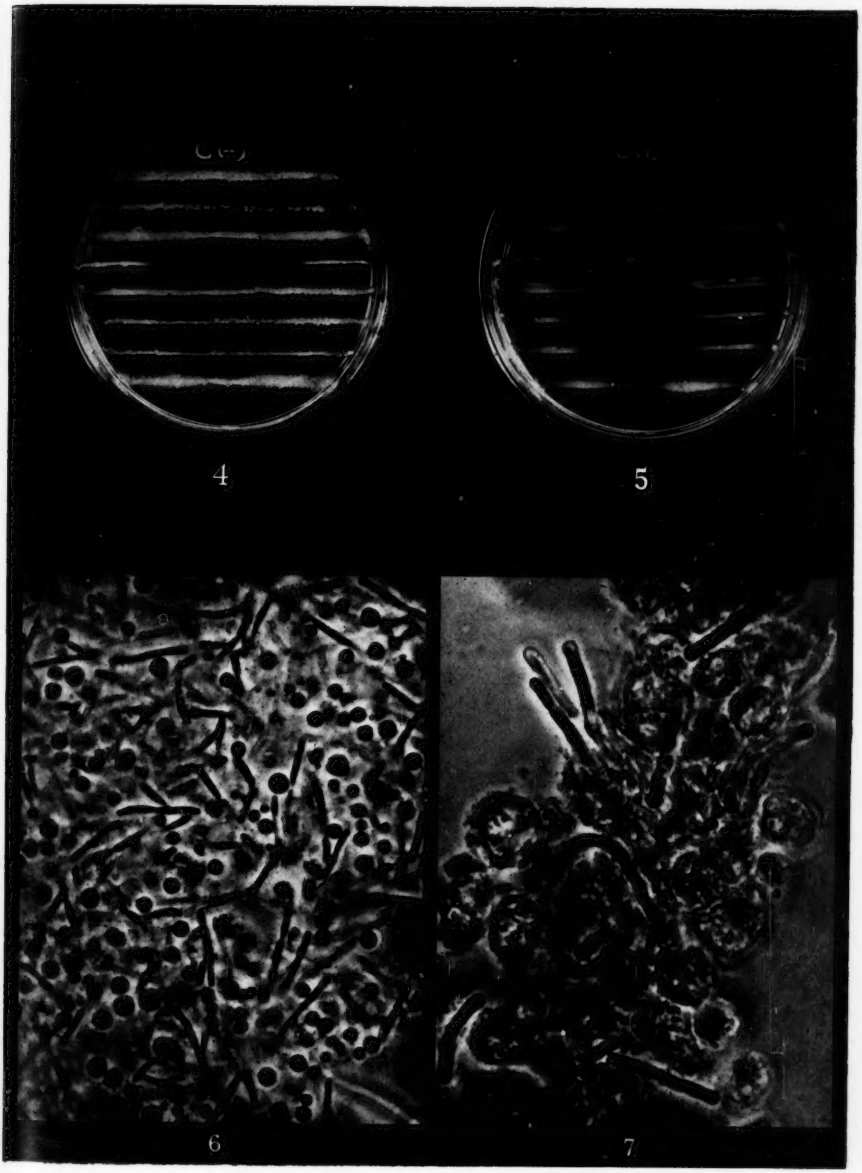
EXPLANATION OF PLATE.

FIG. 4.—Plate with 1/2000 lysozyme in gutter streaked with various strains of *B. anthracis* and incubated in air. The streak showing inhibition is *M. lysodeikticus* which was included as a control.

FIG. 5.—As Fig. 4 but incubated in 20 per cent CO_2 with 0·1 M-$NaHCO_3$ in the medium.

FIG. 6.—The action of 1/2000 lysozyme on a susceptible strain of *B. anthracis* grown in 20 per cent CO_2 with 0·1 M-$NaHCO_3$ in the medium observed after 30 min. at 37°. (Wet preparation under phase contrast. × 800.)

FIG. 7.—Opsonic action of lysozyme on *B. anthracis*. For details see text. (Wet preparation under phase contrast. × 800.)



4 5

6 7

Gladstone and Johnston,

d-glutamic acid capsule, which is produced by *B. anthracis* when grown in CO_2, and lysozyme which is basic. Similarly, a heavy precipitate develops when free poly-*d*-glutamic acid is added to a solution of lysozyme.

It was not possible, therefore, to assess lysozyme action on *B. anthracis* by turbidimetric methods. Two alternative methods were used: inhibition of growth by lysozyme added at the time of inoculation, and assessment of the fall in viable count after the addition of lysozyme to a grown culture.

Growth inhibition by lysozyme.

Lysozyme in twofold dilutions (1/125 to 1/128,000) was added to two series of tubes containing culture medium which were then inoculated with strain B. One series was incubated in air without further addition; the other, to which 0·1M-$NaHCO_3$ had been added, in 20 per cent CO_2. Growth was recorded after 15 hr. Results showed that while there was no inhibition of growth in air, growth was completely inhibited at a lysozyme dilution of 1/32,000 and partially inhibited at a dilution of 1/64,000 in the series incubated in 20 per cent CO_2. A similar test with strain A showed that no inhibition of growth occurred in either case. On the other hand, growth of *Micrococcus lysodeikticus* was completely inhibited by a lysozyme concentration of $1/10^6$ and partially inhibited at half this concentration, there being no difference between the air and CO_2-grown series.

Conditions Determining Lysozyme Sensitivity in Relation to CO_2/HCO_3' Concentrations.

Optimum CO_2/HCO_3' concentration for inhibition by lysozyme.

This was ascertained by preparing several series of cultures as in the previous section. Each contained a different concentration of $NaHCO_3$ and was incubated in the concentration of CO_2 necessary to give a pH of 7·5 when in equilibrium. The results of such an experiment are shown in Fig. 1. Here it can be seen that maximum inhibition of growth occurs in a medium containing 0·11 M-$NaHCO_3$ in equilibrium with 25 per cent CO_2. Control tubes without lysozyme showed that when the amount of CO_2 was raised above 20 per cent, growth was markedly inhibited. Tests above this concentration, therefore, are not valid, and it would appear that 0·1 M-$NaHCO_3$ at pH 7·5 is the optimal concentration for lysozyme action.

Effect of pH at different concentrations of $NaHCO_3$.

Experiments similar to that described above were carried out at pH 7·0, 7·5 and 8, at each of three concentrations of $NaHCO_3$. From Fig. 2 it can be seen that the phenomenon of growth inhibition by lysozyme is entirely dependent on the concentration of $NaHCO_3$ in the medium and is relatively insensitive to changes in pH and CO_2 concentration except where these determine the HCO_3' concentration. Thus, whereas at pH 7·0 with 0·05 M-HCO_3' the cultures have to be incubated in 30 per cent CO_2, at pH 8 with a similar concentration of HCO_3' only 4 per cent CO_2 is required.

Is $NaHCO_3$ necessary for lysozyme action?

The experiments described above show that lysozyme inhibits the growth of susceptible organisms only when $NaHCO_3$ is present in the medium. In such

tests there is no indication as to whether this substance is necessary for the action of lysozyme, or whether it exerts its effect as a constituent of the growth medium in producing a susceptible organism. For this reason the effect of lysozyme was tested on " resting " suspensions of susceptible organisms which had been grown under various conditions.

Strain B was grown in air (without $NaHCO_3$) and in 20 per cent CO_2 (with 0·1 M-$NaHCO_3$) for 15 hr. The organisms were then centrifuged, washed once and re-suspended in 1 per cent Bacto-Vitamin Free Casamino Acids in 0·07 M phosphate buffer at pH 7·5, and standardised by opacity measurements to contain approximately 5×10^7 organisms per ml. Each suspension was divided into four parts. These were treated as follows :

A : No addition ; gassed with air and sealed. B : With 0·1 M-$NaHCO_3$; gassed with 20 per cent CO_2 and sealed. C : With 1/2000 lysozyme ; gassed with air and sealed. D : With 1/2000 lysozyme and 0·1 M-$NaHCO_3$; gassed with 20 per cent CO_2 and sealed.

The suspensions were incubated at 37°, viable counts being made immediately and at intervals over a total period of 5 hr.

The results (Fig. 3) show that lysozyme can exert a bactericidal action in the absence of $NaHCO_3$ although the effect is more marked in its presence. The main action of $NaHCO_3$ in making the organisms susceptible to lysozyme would appear, therefore, to take place during the growth of the cell. Cells grown in air are not susceptible even when $NaHCO_3$ is present in the test system.

Lysozyme Sensitivity and Capsulation.

It is well known that when *B. anthracis* is grown in the presence of added CO_2 it forms capsules. This suggested that the presence of capsules might be a necessary adjunct for lysozyme action, particularly since, as stated above, there was an evident reaction between the capsular substance and lysozyme. However, a number of observations showed that this was not the case :—(*a*) Well marked capsulation of B was obtained when the organisms were grown in 5 per cent CO_2 with 0·025 M-$NaHCO_3$ in the medium but such organisms were not susceptible to lysozyme (Fig. 3). (*b*) When B was grown at 42° in 20 per cent CO_2 with 0·1 M-$NaHCO_3$ no capsules were formed. The organisms, however, showed the same sensitivity to lysozyme as those grown at 37°. (*c*) Strain B/R, the rough variant of B, failed to form capsules under any conditions of growth. This strain showed the same susceptibility to lysozyme as did the parent strain and the conditions necessary for this susceptibility were in all respects similar.

Lysozyme Susceptibility and the Production of Soluble " Protective Antigen ".

It has been shown that $NaHCO_3$ is required for the production of " protective antigen " (Gladstone, 1948). There seems, however, to be no relationship between lysozyme susceptibility and the ability to produce this antigen. B and B/R produced the antigen as did many of the strains insensitive to lysozyme action.

Microscopical Observation of the Action of Lysozyme on Strain B.

When 1/2000 lysozyme is added to a suspension of strain B grown under conditions appropriate for maximum sensitivity and examined as a wet preparation

under phase contrast, the first change noted is in the capsules. Normally the capsule cannot be observed with phase contrast. The addition of lysozyme causes it to become opaque, giving a pseudo-capsular reaction similar to that observed by Tomcsik and Guex-Holzer (1954). They showed that this was not specific for lysozyme ; the same reaction was observed with other basic proteins such as protamine.

This pseudo-capsular reaction was found to occur with all capsulated strains of *B. anthracis* tested. It appears to have no relationship to lysozyme sensitivity.

The subsequent action on sensitive strains appeared to be at localised areas on the wall of the organism, particularly at the ends of the cells. At these points " herniation " of the contents of the cell was observed to take place in the form of spherical bodies, which maintained their shape for a considerable time after extrusion from the cell (Fig. 6). These clearly correspond to the structures observed by Weibull (1953) when *B. megatherium* is lysed by lysozyme, but they were not so sensitive to variations in osmotic pressure. In the case of *B. megatherium* the action of lysozyme appears to involve the whole of the cell wall ; with *B. anthracis* it is confined to localised areas. The spherical bodies gradually became less opaque and finally disappeared while the cell, emptied of its contents, remained for some time as a " ghost " until it also disintegrated, leaving an amorphous mass of poorly defined débris.

Lysozyme is not entirely without action on strains which are not destroyed by the enzyme. Chains and pairs of capsulated organisms of the insensitive strain A tend to be broken up into single organisms. This occurs without any accompanying loss of viability.

The Opsonic Action of Lysozyme on Capsulated Strains of B. anthracis.

The capsule, while not the sole factor in determining the virulence of *B. anthracis*, is undoubtedly the factor which prevents phagocytosis. The following experiment was carried out in order to test whether lysozyme could act as an opsonin by its union with the capsule.

A suspension of leucocytes was obtained by injecting a guinea-pig intraperitoneally with 100 ml. saline. The cells were centrifuged and re-suspended in normal guinea-pig serum. Capsulated organisms of strains A and B were each suspended in saline and standardised to 10^7 organisms per ml. One ml. of each of these suspensions was added to an equal volume of the leucocyte suspension and rotated at 37° in waxed bottles. After 1 hr. the suspensions were examined microscopically using phase contrast. Not a single organism of either strain A or B had been phagocytosed in 200 leucocytes counted.

A similar experiment was carried out except that 1/2000 lysozyme in normal saline was added to each suspension. Clumping of leucocytes and bacteria occurred in a few minutes, the latter showing the pseudo-capsular reaction mentioned above. After 30 min. a considerable number of organisms of both the sensitive strain B and the insensitive strain A were engulfed, many of them showing signs of disintegration (Fig. 7). In addition with strain B extracellular disintegration was observed. Thus opsonic action appears to be independent of lysozyme sensitivity and is probably determined by the reaction between lysozyme and the capsule. Other basic proteins such as protamine (Gordon and Thompson, 1937) and globin (Gordon and Atkin, 1939) have been described as opsonins.

Lysozyme Sensitivity in Relation to Virulence.

There seems to be little relationship between the sensitivity of strains of *B. anthracis* to lysozyme and their virulence. All the sensitive strains with the exception of the non-capsulated B/R were equally virulent for mice, and showed in this capacity little difference from certain of the insensitive strains. On the other hand, they differed considerably in their virulence for guinea-pigs. The sensitive strain D was approximately as virulent as the insensitive strain A, although the sensitive strain B was only about one tenth as virulent.

In spite of the fact that the virulence of sensitive strains for mice is no different from that of insensitive strains, it may be considerably reduced if lysozyme is injected with the challenge dose. The results presented in the Table show that the virulence of the sensitive strain B is reduced tenfold when 1/2000 lysozyme is added to the inoculum. In contrast, the virulence of the insensitive strain A is unaffected.

TABLE.—*The Effect of Lysozyme on Virulence of Strains A (Insensitive) and B (Sensitive) in Mice.*

Strain.	Number viable spores injected.	Deaths. (Number dead/number tested.) Without lysozyme.	With 1/2000 lysozyme.
A	45,000	10/10	9/10
	4,500	9/10	8/10
	450	4/10	6/10
	45	0/10	0/10
	Total	23/40	Total 23/40
B	30,000	29/30	23/30
	3,000	20/30	12/30
	300	11/30	2/30
	30	2/30	0/30
	Total	62/120	Total 37/120

Mice (20–22 g.) were injected i.p. with spores suspended in 0·5 ml. saline. Deaths were recorded after 7 days.

DISCUSSION.

The results recorded in this paper demonstrate one of the few cases of a pathogenic organism being highly sensitive to lysozyme. This sensitivity, however, is only found when the organism is grown in a rather high concentration of HCO_3' and CO_2. Under such conditions the organism resembles that growing in the animal body in that it produces a capsule and protective antigen. However, neither of these factors appears to determine sensitivity and the HCO_3' concentration in normal serum and body tissues (about 0·025 M) is below that required for lysozyme sensitivity, as demonstrated *in vitro*. In spite of this, there does appear to be some sensitivity *in vivo*, as shown by direct observation of the action of lysozyme on organisms of strain B taken from the peritoneum of an infected guinea-pig and the reduction in virulence for mice brought about by the injection of lysozyme at the time of challenge.

Although the phenomenon of dependence of growth conditions on lysozyme sensitivity has only been demonstrated with a few strains of *B. anthracis*, it

raises once again the question as to how far lysozyme may not have some protective action in infections caused by pathogenic organisms.

We have made a few experiments to test whether other pathogens, not normally lysozyme-sensitive, may be made so by growth in the presence of relatively high concentrations of HCO_3' and CO_2. Three laboratory strains of pneumococcus and three of Group A haemolytic streptococci were grown on plates made from CCY agar incorporating 20 per cent serum and 0·1 M-$NaHCO_3$ with 1/2000 lysozyme in a gutter. After incubation in 20 per cent CO_2 it was found that no organism was inhibited.

The demonstration that lysozyme can act as an opsonin, even when the organism is not susceptible to its lytic action, suggests that it has the same action *in vivo*. However, although capsulated organisms of the insensitive strain A were rapidly phagocytosed when injected together with 1/2000 lysozyme intraperitoneally into guinea-pigs in which a local saline-induced leucocytosis had been previously produced, the animals survived no longer than did the controls.

It is probable that HCO_3' and CO_2 are necessary for the synthesis of the lysozyme substrate in the cell walls of the sensitive strains. This substrate appears to be situated at localised areas in the cell wall, at the ends and at points along the cell probably corresponding to the septa formed before division takes place. If so, it is possible that this substrate is present in sensitive and insensitive strains alike when grown in the appropriate concentrations of HCO_3' and CO_2. In sensitive strains the presence of the substrate determines the integrity of the cell wall, while with insensitive strains the action of lysozyme merely has the effect of breaking up chains of organisms, perhaps by acting on substrate present in the capsule. There is evidence for the presence of localised areas of polysaccharide in the capsule of *B. anthracis* (Tomcsik 1954). Such areas may represent the site of the lysozyme substrate.

Various problems arising out of the findings in this paper are being studied, and in particular the effects of weak acids in replacing bicarbonate in the production of lysozyme sensitivity in synthetic media. The results of these investigations will appear in subsequent publications.

SUMMARY.

Thirteen strains of *B. anthracis* were tested for sensitivity to lysozyme. No strain was found to be susceptible when grown under ordinary laboratory conditions in air. Five strains were highly susceptible when cultured in 10–20 per cent CO_2 in a medium containing 0·05–0·1 M-HCO_3'.

The rôle of CO_2 and HCO_3' in this phenomenon was investigated. The phenomenon does not appear to depend on capsulation of the organisms, and bears no relationship to their virulence or power to produce protective antigen.

Our thanks are due to Dr. D. W. Henderson and Dr. M. Sterne for strains of *B. anthracis*, and to Dr. G. Alderton of Western Regional Research Laboratory, Albany, California, U.S.A. for a generous sample of purified lysozyme.

REFERENCES.

ABRAHAM, E. P. AND ROBINSON, R.—(1937) *Nature, Lond.*, **140**, 24.
AMANO, T., KATO, K. AND SHIMA, M.—(1953) *Med. J. Osaka Univ.*, **3**, 695.

GLADSTONE, G. P.—(1948) *Brit. J. exp. Path.*, **29**, 379.
Idem AND FILDES, P.—(1940) *Ibid.*, **21**, 161.
GORDON, J. AND ATKIN, W. R.—(1939) *J. Path. Bact.*, **48**, 477.
Idem AND THOMPSON, F. C.—(1937) *Brit. J. exp. Path.*, **18**, 390.
MELNIK, M. I.—(1936) " Lysozyme, Typhoid Fever, Diphtheria and Rheumatism ", Kharkoff (Monographs Metchnikoff Institute).
MILES, A. A. AND MISRA, S. S.—(1938) *J. Hyg., Camb.*, **38**, 732.
STERNE, M.—(1937) *Onderstepoort J. vet. Sci.*, **8**, 271.
TOMCSIK, J.—(1954) *Schweiz. Z. allg. Path.*, **17**, 457.
Idem AND GUEX-HOLZER, S.—(1954) *J. gen. Microbiol.*, **10**, 97.
WEIBULL, C.—(1953) *J. Bact.*, **66**, 688.
WOLFF, L. K.—(1927) *Z. ImmunForsch.*, **54**, 188.
WRIGHT, G. G., HEDBERG, MARY A. AND FEINBERG, R. J.—(1951) *J. exp. Med.*, **93**, 523.

THE NEUROTOXIN OF *SHIGELLA SHIGAE*.

5. The Effect of Iron and Related Metals on the Growth, Respiration and Toxin Production of *Shigella shigae* and Related Organisms.

W. E. van HEYNINGEN.

From the Sir William Dunn School of Pathology, University of Oxford.

Received for publication April 6, 1955.

An excess of iron in the culture medium of *Shigella shigae* causes a diminished production of neurotoxin (Dubos and Geiger, 1946). A preliminary study of this phenomenon was made during work on the purification of the toxin (van Heyningen and Gladstone, 1953*a*, *b*), and this has now been followed by a further investigation of the effect of iron and the related metals, cobalt and nickel, on the growth, respiration and toxin production of *Sh. shigae*. These effects have been compared with those on related organisms which do not produce a neurotoxin, *Shigella flexneri*, *Shigella sonnei* and *Bacterium coli*. Although these studies have, so far, thrown little light on the mechanism of toxin production they are of interest for other reasons.

METHODS.

The rough strain of *Sh. shigae*, the de-ferrated culture medium, and the methods for estimating growth, and for extracting and estimating the toxin by flocculation have been described in previous papers (van Heyningen and Gladstone, 1953*a*, *b*). The product of the values, Lf per ml. toxic extract and flocculation time in min. (Lf. Kf), continues to be a reliable guide to the true flocculation zone. Flocculation assays are accepted only when the Lf. Kf is 1000 ± 200. Rough strains of *Sh. flexneri*, *Sh. sonnei* and *Bact. coli* were used. Following the suggestion of Monod (1949), the rate of growth is shown by plotting the logarithm to the base 2 of the dry weight of organism per ml. against time, so that a unit increase in $\log_2$ mg. organism per ml. corresponds to a doubling of growth, or one division. Tables of logarithms to the base 2 were kindly provided by Dr. D. J. Finney, and have since been published (Finney, Hazlewood and Smith, 1955). Cultures were grown either in 10 ml. volumes in modified Monod T tubes (van Heyningen and Gladstone, 1953*b*) or in 1 l. volumes in 5 l. Mitchell (1949) whirling flasks. The de-ferrated medium contained approximately 0·1 μg. iron per ml., and correspondingly low concentrations of cobalt and nickel, but these were not measured. Iron was added in the form of ferrous ammonium sulphate (British Drug Houses Ltd., Analar), with an equal weight of citric acid; cobalt was added as cobalt chloride (Kahlbaum) and nickel as nickel chloride (British Drug Houses Ltd., Analar). Magnesium was added to all media in a concentration of 10 μg. per ml. Respiration of washed resting suspensions of cells was measured in Warburg manometers. Substrate was tipped in from side bulbs after equilibration to give a final concentration of 0·018 M. All readings are corrected for endogenous respiration.

EXPERIMENTAL.

Growth Rates.

Cultures from slopes of tryptic meat agar were inoculated into T tubes containing de-ferrated medium to which the following additions had been made: (*a*)

nothing, (*b*) 1 μg. Fe per ml., (*c*) 10 μg. Co per ml., (*d*) 20 μg. Ni per ml. These new cultures were grown for 6 hr. at 37°, stored at 4° overnight, and then inoculated into fresh T tubes containing the same media. The cultures were incubated at 37° and growth was estimated at intervals of 30 min. The results are shown in Fig. 1, 2 and 3. In the case of *Sh. shigae* the growth rate in iron-rich medium is

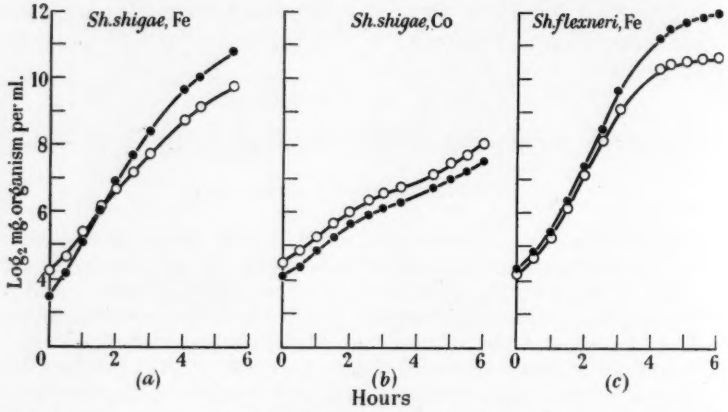


FIG. 1.—Effects of Fe (*a* and *c*) and Co (*b*) on growth rates of *Sh. shigae* (*a* and *b*) and *Sh. flexneri* (*c*). ● = metal-containing medium ; ○ = metal-deficient medium.

faster than in metal-deficient medium (Fig. 1*a*), or in media containing high concentrations of cobalt (Fig. 1*b*) or nickel. The effect of iron deficiency is seen more clearly when an iron-poor inoculum is used (Fig. 2*a*). In this case the rate of

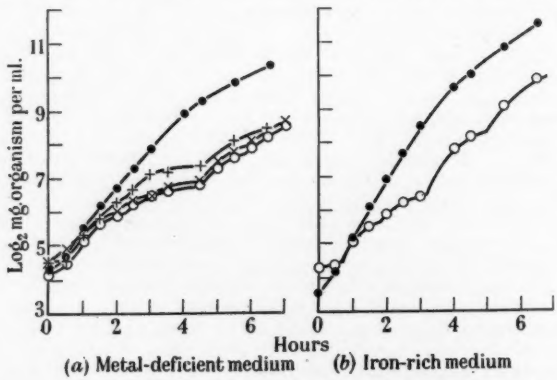


FIG. 2.—Growth rates of metal-deficient (○), iron-rich (●), cobalt-rich (×), and nickel-rich (+) inocula in metal-deficient (*a*) and iron-rich (*b*) media.

growth falls off and recovers periodically in a manner reminiscent of Monod's (1949) *diauxie*, and the effect is seen even when the iron-poor inoculum is placed in an iron-rich medium (Fig. 2*b*).

The growth rates of the related organisms, *Sh. flexneri* (Fig. 1c), *Sh. sonnei* and *Bact. coli* are apparently unaffected by the iron content of the medium, or of the inoculum (Fig. 3*a*, *b*).

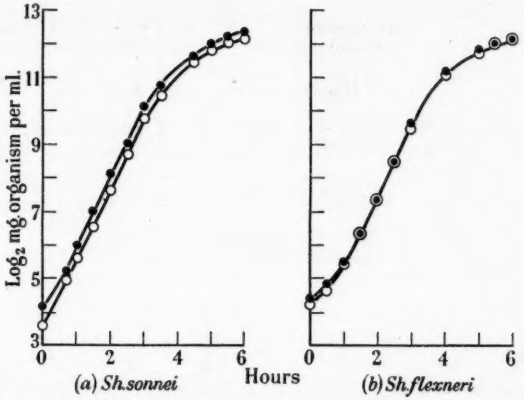


FIG. 3.—Growth rates of metal-deficient (○) and iron-rich (●) inocula of *Sh. sonnei* (*a*) and *Sh. flexneri* (*b*) in metal-deficient medium.

Growth Yields.

As far as growth rate is concerned, iron is the only metal a deficiency of which leads to retardation, and *Sh. shigae* the only organism affected. The growth yield of all four organisms, on the other hand, is increased by all three metals, as can be seen in Tables I and II. Table II also shows that only in the case of *Sh. shigae* does the concentration of iron in the inoculum have a significant effect on the growth of the culture. Fig. 4*a* shows that in different experiments (A, B, C, D) great variations in growth yield may take place in media containing the same concentration of iron. These variations in growth affect the iron content of the cells since all the iron in this range of concentrations is taken up by the organisms (van Heyningen and Gladstone, 1953*b*; van Heyingen), 1955, and this in turn affects the toxin content of the cells.

TABLE I.—*Effect of Various Metals in the Culture Medium on Growth Yield and Toxicity of* Sh. shigae.

Metal.	mg. Organism per ml.	Lf per mg. organism.
0 (*a*)	1·5	2·0
0 (*b*)	1·3	2·7
1 μg. Fe per ml. (*a*)	2·6	0·3
1 μg. Fe per ml. (*b*)	3·0	0·3
10 μg. Co per ml. (*a*)	2·2	<0·3
10 μg. Co per ml. (*b*)	2·9	<0·3
20 μg. Ni per ml. (*a*)	1·7	0·9
20 μg. Ni per ml. (*b*)	1·9	2·4

a and *b* refer to separate experiments.

TABLE II.—*Effect of Iron Content of Inocula and of Media on Growth Yields of Various Organisms.*

Organism.	Fe content of inoculum.	mg. Organism per ml. 0·1 μg. Fe per ml. medium.	1·0 μg. Fe per ml. medium.
Sh. shigae	Low	0·5	0·8
	High	1·3	2·7
Sh. flexneri	Low	1·5	4·5
	High	1·6	4·5
Sh. sonnei	Low	1·9	4·5
	High	2·1	4·9
Bact. coli	Low	2·1	4·2
	High	2·4	5·0

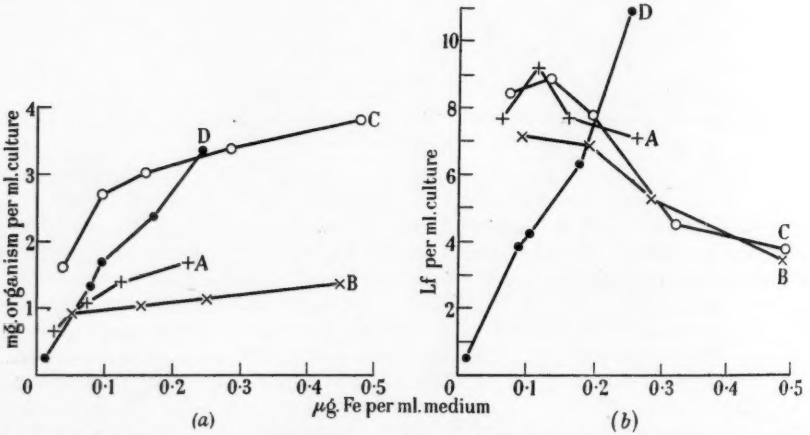


FIG. 4.—Effect of iron content of medium on (*a*) growth yield and (*b*) toxin production in *Sh. shigae*. A, B, C and D represent different experiments.

Toxin Production.

Within the range of iron concentrations considered in this and in previous papers the toxin content of a culture generally decreased with increasing iron content, as can be seen in cases A, B and C, Fig. 4*b*. In the case D growth yield was a linear function of the iron content of the medium; the iron and toxin contents of the cells were thus constant and the total toxin content of the culture therefore increased with the increasing yield of organisms. Subsequent studies (van Heyningen, 1955) have shown that at low iron concentrations the toxin content of the cells may remain constant even when the iron content increases, as long as all this iron is in the form of haems.

Table I shows that cobalt also inhibits toxin production if present in ten times the concentration at which iron is inhibitory. The effect of nickel is variable, as it is with growth yield.

The effect of iron on growth yield was observed with all the four organisms tested; the effect on growth rate was observed only with *Sh. shigae* which is also the only organism producing neurotoxin. This would suggest that the stimulatory effect of iron on growth rate might be connected with its inhibitory effect on toxin production, were it not for the fact that cobalt, which has no effect on growth rate, inhibits toxin production (see Fig. 1*b*, 2*a* and Table I).

Respiration.

It had been shown previously that toxic organisms grown in an iron-poor medium oxidised succinate and other Krebs cycle intermediates only at a low rate, but substrates such as glucose, pyruvate and lactate at a high rate, whereas non-toxic organisms grown in an iron-rich medium oxidised all these substrates at a high rate (van Heyningen and Gladstone, 1953*b*). The difference in behaviour towards Krebs cycle intermediates between iron-rich and iron-poor *Sh. shigae* resembles the difference between cultures of *Pasteurella pestis* grown aerobically

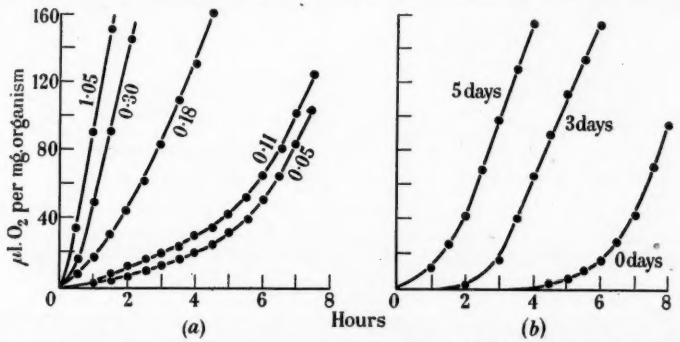


FIG. 5.—Oxygen uptake by washed resting suspensions of *Sh. shigae* (*a*) grown in media of various iron contents (figures on curves are μg. Fe/ml.), and (*b*) grown in medium containing 0·05 μg. Fe per ml. and stood as washed suspension at room temperature for various days.

and anaerobically (Englesberg, Gibor and Levy, 1954; Englesberg, Levy and Gibor, 1954). The inability of anaerobically grown *P. pestis* to oxidise Krebs cycle intermediates was ascribed to an absence of iron-containing cytochrome, but such an explanation does not hold in the case of iron-poor *Sh. shigae*. It has been shown that all the iron in iron-poor medium is converted into haem by this organism, and that cells which are apparently unable to oxidise Krebs cycle intermediates contain at least half as much cytochrome as iron-rich cells (van Heyningen, 1955).

It was noticed that when iron-rich organisms oxidised Krebs cycle intermediates there was a lag before the rate of oxidation reached its maximum (see Fig. 5, 6, 7, 8). This suggested the possibility that the inability of iron-poor organisms to oxidise these substrates might only be apparent, and that they might oxidise them vigorously after a longer lag period than that seen with iron-rich organisms. Fig. 5*a* shows that this indeed is the case. The length of the lag period is inversely proportional to the iron content of the organism, and with organisms grown in very low concentrations of iron (0·01 μg. Fe per ml.) more

than 10 hr. may pass before there is an appreciable uptake of oxygen. Iron-poor organisms can be made to behave like iron-rich organisms by allowing washed resting suspensions of the cells to stand in phosphate buffer, pH 7·8, at room temperature for some days. Fig. 5*b* shows that the lag period then progressively diminishes. Although the oxidative behaviour of toxic organisms was changed in this way to that of non-toxic organisms there was no concomitant decrease in their toxicity. Observations similar to those with succinate were also made with other Krebs cycle intermediates, *viz.*, citrate, *cis*-aconitate, α-ketoglutarate, fumarate, malate and oxalacetate.

In an attempt to investigate the nature of the lag the following experiment was done with iron-rich organisms that oxidised fumarate with a lag of about 20 min. After equilibration a small amount of fumarate was added to the cells and the oxygen uptake followed; within 80 min. the substrate was completely exhausted and no further oxygen was consumed. At 102 min. a further amount of fumarate was added and oxygen uptake proceeded immediately at its maximum rate, without lag, as can be seen in Fig. 6*a*. However, when 100 min. were

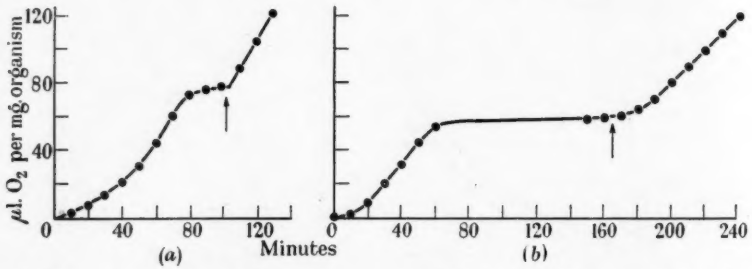


FIG. 6.—Oxygen uptake by washed resting suspension of iron-rich *Sh. shigae* after addition of fumarate at zero time and at times indicated by arrows. Lag abolished in (*a*) but not in (*b*).

allowed to elapse between the exhaustion of the first amount of fumarate and the addition of a second amount a lag appeared again (Fig. 6*b*). Similar results were observed with succinate and malate, the lag in the latter case never being completely abolished, but only diminished. The lag in the oxidation of fumarate was also abolished in organisms immediately after the exhaustive oxidation of succinate, but not after malate.

The oxidative behaviour of washed suspensions of resting iron-deficient organisms could not be altered by the addition of iron to the suspending fluid, or by drying the cells, or by repeatedly freezing and thawing them; nor could iron-rich cells be made to behave like iron-poor cells by treating them with the chelating agent ethylene diamine tetra-acetic acid.

Iron-poor *Sh. flexneri* (Fig. 7*b*), *Sh. sonnei* and *Bact. coli* showed similar lags in the oxidation of Krebs cycle intermediates, and these lags could also be reduced by standing washed suspensions of the organism at room temperature.

Fig. 7 shows that the differences in oxidative behaviour between iron-poor and iron-rich organisms could only be demonstrated when the organisms were grown in Mitchell flasks. When they were grown in Monod T tubes the rate of oxidation of Krebs cycle intermediates by iron-poor organisms was increased, and that by

iron-rich organisms diminished, with the result that the difference between the organisms was barely observable.

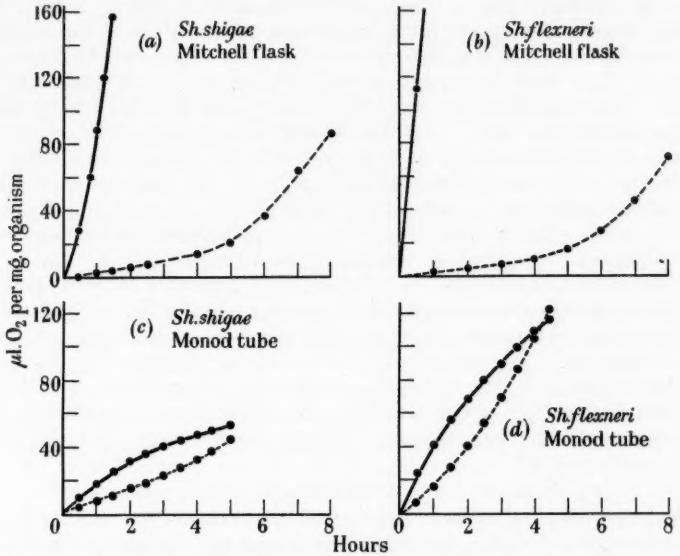


FIG. 7.—Oxidation of succinate by washed resting suspensions of *Sh. shigae* (*a* and *c*) and *Sh. flexneri* (*b* and *d*) grown in Mitchell flasks (*a* and *b*) and Monod tubes (*c* and *d*) in iron-poor (broken lines) and iron-rich (unbroken lines) medium.

The toxicity of the organism does not appear to bear any direct relation to its oxidative behaviour. On numerous occasions the same inoculum was grown in the same iron-poor medium in both Mitchell flasks and Monod tubes and in none of them was any significant difference in toxicity found. Moreover, the addition to iron-deficient medium of cobalt and nickel, which inhibit toxin production, has little or no effect on the oxidative behaviour of the organism (Fig. 8).

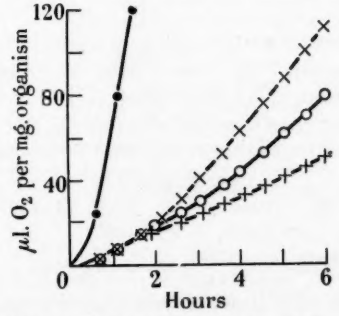


FIG. 8.—Oxidation of fumarate by washed resting suspension of *Sh. shigae* grown in de-ferrated medium with addition of nothing (○), 1 µg. Fe per ml. (●), 10 µg. Co per ml. (×), 20 µg. Ni per ml. (+).

DISCUSSION.

The object of the experiments reported in this paper was to investigate the mechanism of the inhibition of toxin production in *Sh. shigae* by iron. Although several interesting observations have been made of the effect of this metal on the growth characteristics and respiratory activity of the organism, we have seen that it is unlikely that they have any direct bearing on the mechanism of toxin production. This being so, they have not, so far, been followed up in great detail.

For the present we are unable to suggest any explanation for the delayed growth-rate of metal-deficient *Sh. shigae*, or why this organism should differ in this respect from the related organisms. Nor can we explain why cobalt and nickel, which are like iron in affecting growth yield and toxin production, should not have the same effect as iron on growth rate or respiratory behaviour.

A lag in the rate of oxidation of Krebs cycle intermediates, and its diminution when the organism has had previous contact with members of the cycle, has been observed by a number of workers with various organisms. The formation of adaptive enzymes to account for these lags has been considered, although it is difficult to envisage a mechanism that would be appropriate to the complex of enzymes that operate in the Krebs cycle (see Kogut and Podoski, 1953). The observation that the lag may be prolonged in iron-deficient organisms is new, and although it appears to have little connexion with the mechanism of toxin production work on it continues.

SUMMARY.

The growth rate of *Sh. shigae*, but not of *Sh. flexneri*, *Sh. sonnei*, or *Bact. coli*, is retarded in metal-deficient medium. Iron, but not cobalt or nickel, increases the growth rate of *Sh. shigae*. Iron, cobalt and nickel have a stimulatory effect on the yield of growth of *Sh. shigae*, *Sh. flexneri*, *Sh. sonnei* and *Bact. coli*. Iron, cobalt and nickel have an inhibitory effect on neurotoxin production in *Sh. shigae*.

Sh. shigae, *Sh. flexneri*, *Sh. sonnei* and *Bact. coli*, when grown in metal-deficient medium, show considerable lags in the oxidation of Krebs cycle intermediates. These lags are nearly abolished when the organisms are grown in the presence of iron, but not of cobalt or nickel. The appearance of the lag depends also on the method by which the cultures are grown.

There appears to be little connexion between the effects of the metals on the growth and respiratory characteristics of these organisms and on toxin production in *Sh. shigae*.

Work on this toxin would be impossible without the dysentery antitoxin so generously and freely supplied by Miss Mollie Barr and Mrs. I Batty of the Wellcome Research Laboratories, Beckenham. I am indebted further to Dr. R. J. P. Williams for helpful advice and discussion, and, as usual, I am grateful to Miss Winifred Crawford for highly competent technical assistance.

REFERENCES.

DUBOS, R. AND GEIGER, J. W.—(1946) *J. exp. Med.*, **84**, 143.
ENGLESBERG, E., GIBOR, A. AND LEVY, J. D.—(1954) *J. Bact.*, **68**, 146.
Idem, LEVY, J. D. AND GIBOR, A.—(1954) *Ibid.*, **68**, 178.
FINNEY, D. J., HAZLEWOOD, T. AND SMITH, M. J.—(1955) *J. gen. Microbiol.*, **12**, 222.
KOGUT, M. AND PODOSKI, E. P.—(1953) *Biochem. J.*, **55**, 800.
MITCHELL, P.—(1949) *Nature, Lond.*, **164**, 846.
MONOD, J.—(1949) *Annu. Rev. Microbiol.*, **3**, 371.
VAN HEYNINGEN, W. E.—(1955) *Brit. J. exp. Path.*, **36**, **381**.
Idem AND GLADSTONE, G. P.—(1953*a*) *Ibid.*, **34**, 202.—(1953*b*) *Ibid.*, **34**, 221.

THE NEUROTOXIN OF *SHIGELLA SHIGAE*.

6. The Relation between Toxin and Cytochrome Production in the Organism.

W. E. van HEYNINGEN.

From the Sir William Dunn School of Pathology, University of Oxford.

Received for publication April 6, 1955.

Both in *Shigella shigae* and in *Corynebacterium diphtheriae* toxin production diminishes and the cellular content of iron-containing cytochrome pigments increases when the organism is grown in iron-rich medium. In the case of the diphtheria bacillus Pappenheimer (1955) has suggested that the toxin may be related to the protein moiety of the bacterial cytochrome *b*, and that it is secreted in the unconjugated state by the organism when there is not sufficient iron present to form haem from porphyrin and in this state to combine with the protein to form cytochrome. According to this hypothesis toxin formation takes place at the expense of haem formation. It has now been shown that the contrary is true in the case of *Sh. shigae*; neurotoxin production is at its highest when the synthesis of haems is most efficient.

METHODS.

Cultures.

The CCY medium of Gladstone and Fildes (1940) which had been used hitherto (van Heyningen and Gladstone, 1953*a*) was modified by replacing the acid hydrolysate of casein with 1 per cent (w/v) Bacto Casamino acids (Difco Laboratories, Michigan), the tryptic digest of casein with 0·0002 M tryptophane, and the yeast extract with 1 per cent (w/v) Bacto yeast extract (Difco). A twentyfold concentrated stock was de-ferrated by three successive precipitations of calcium phosphate in the manner previously described (van Heyningen and Gladstone, 1953*b*), except that the calcium phosphate precipitates were removed by centrifugation rather than filtration. Before use the concentrate was diluted 1/10 with distilled water, 10 μg. Mg (as magnesium sulphate) were added per ml., iron (as ferrous ammonium sulphate with an equal weight of citric acid) was added as required, the pH was adjusted to 7·4 and the medium was autoclaved. The iron content of the diluted de-ferrated medium varied from 0·01 to 0·03 μg. Fe per ml. These values are somewhat lower than those previously reported, probably because the filter paper then used to remove the calcium phosphate precipitate contained an appreciable amount of soluble iron.

The organism was the Dubos R strain previously employed. It was grown overnight at 37° in 1 l. volumes of medium in 5 l. Mitchell (1949) whirling flasks.

Estimation of Iron.

The estimation of iron at the very low concentrations in which it is present in the de-ferrated medium, even before dilution, presented a problem. It was hoped that it could be overcome by dry-ashing a sufficiently large volume of the tenfold concentrated stock by the method of Woiwod (1947). However, it was found that a volume of concentrate (*ca.* 20 ml., or 8 g. solids) that contained a measurable amount of iron contained also so much ash that it interfered grossly with the measurement. Eventually it was found that a modification of

the oxine-extraction method of Moeller (1943) was reasonably accurate and convenient. This depends on extracting the sample with a solution of oxine (8-hydroxyquinoline) in chloroform; the iron passes to the chloroform phase to form a green complex with the oxine with an absorption maximum at 580 mμ., the intensity of which is a measure of the iron in the sample. Other metals do not interfere with the estimation.

In order to test the recovery of iron from complex concentrated medium by this technique the following experiment was done. To 40 ml. samples of concentrated de-ferrated medium were added increasing amounts of iron containing a proportion of radioactive iron, Fe-59, and 10 ml. volumes of 0·03 M oxine in chloroform. The samples were extracted for 3 hr. by inverting the mixtures mechanically once a second on a table turning about a horizontal axis. The radioactivity of the aqueous phase and the D_{580} of the chloroform phase were then determined after they had been separated by centrifuging. The results are shown in Fig. 1. Determination of radioactivity showed that the added iron was quantitatively taken up from the aqueous phase. The values for D_{580} of the chloroform phase lie on a

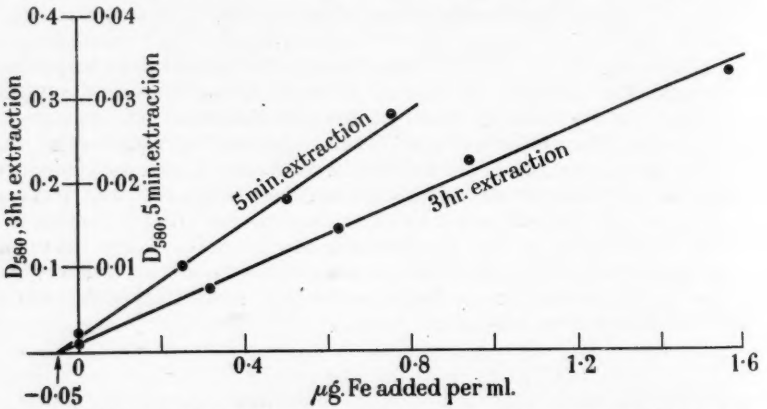


FIG. 1.—Values for D_{580} of chloroform phase after 5 min. and 3 hr. extraction with a solution of oxine in chloroform of samples of media with various amounts of iron added. The intercept with the abscissa of the lines drawn through these points represents the iron content of the medium before the addition of iron.

straight line, and the distance between the origin and the intercept of this line with the abscissa represents the iron content of the medium without added iron. When a similar experiment was done, except that the extraction was continued for 5 min. instead of 3 hr., only a small proportion of the iron was transferred to the chloroform phase, and yet the line passing through the values for D_{580} passed through the same intercept. From the values obtained in the 3 hr. extraction experiment it was calculated that μg. Fe per ml. sample $= 4{\cdot}53 \times D_{580}$ of chloroform-oxine extract, provided the sample is extracted for 3 hr. with 0·25 volume of chloroform-oxine. With other ratios of sample and extractant an appropriate adjustment is made. Estimations of iron in further samples of media and in washed organisms were based on this principle. The method suffers from the weakness that it assumes either that all the iron in the medium is extractable in this way, or that the iron that is not extractable is not available to the organism. Later work showed that haem iron in organisms was not extractable with chloroform-oxine, and also that when haematin was added to the medium the organisms were able to utilise the iron in it. However, the medium normally does not contain haematin and the risks of this method are judged to be justifiable.

Other Estimations.

Bacterial concentration, expressed as mg. dry weight of organism per ml., and toxin, expressed as Lf units, were estimated by methods previously described (van Heyningen and Gladstone, 1953*a*). For estimations of radioactivity 0·2 ml. samples were dried on aluminium

source holders under an infra-red lamp and counted with a thin window Geiger-Müller counter.

EXPERIMENTAL.

Uptake of Iron by Sh. shigae.

In a previous study (van Heyningen and Gladstone, 1953*b*) it was shown that filtrates of cultures grown on media containing up to 1 μg. Fe per ml. contained no detectable iron, and it was concluded that up to this concentration all the iron is taken up by the organism. This conclusion has now been confirmed by experiments with radioactive iron. In most of the experiments reported in this paper the iron added to the de-ferrated medium (never more than 0·8 μg. Fe per ml.) was mixed with a small proportion of radioactive iron, Fe-59. After growth the organisms were spun down, washed with 1 per cent NaCl, made up to a concentration of 40–60 mg. organism per ml., and estimations of toxin, haem, and radioactivity made. At the same time the radioactivity of samples of the medium before inoculation and of the culture filtrate were also determined. Comparisons of the radioactivity of the media and the washed suspensions showed in all cases an apparent uptake of only 60–70 per cent of the iron. However, it appeared that these values were falsely low, owing to self-absorption of radioactivity by the dried organisms. When 50–60 mg. non-radioactive organisms were suspended in radioactive medium and samples dried and placed in the counter the counts were found to be reduced to 60–70 per cent. Moreover, in no case was the radioactivity of a culture filtrate found to be significantly greater than the background count in the instrument. Thus it is clear that within the range of concentrations employed all the iron in the medium is taken up by the organisms, and it is unlikely that any of the iron in the organisms is removable by washing. In all cases, therefore, the value for μg. Fe per mg. organism can be derived from the ratio, μg. Fe per ml. medium : mg. organism per ml. culture.

The Cytochromes of Sh. shigae.

A culture of *Sh. shigae* was grown in a medium containing 1μg. Fe per ml. and the organisms were washed and suspended at a concentration of 50 mg. per ml. in phosphate buffer, pH 7·8. The absorption bands of the suspensions were studied with a low-dispersion spectroscope and a Hartridge reversion spectroscope. The absorption bands correspond with those observed in *Bacterium coli* by Keilin and Harpley (1941): (i) A strong band at 630 mμ. that moved to 635 mμ. when carbon monoxide was bubbled through the suspension, and to 645 mμ. when air was bubbled through; on standing, or on the addition of lactate, the band returned to 630 mμ. and on addition of alkali it disappeared irreversibly. This band is due to cytochrome a_2. (ii) A just perceptible band at 590 mμ. corresponding to cytochrome a_1. (iii) An intense fairly broad band centred at 560 mμ. which disappeared on shaking and re-appeared on standing, or on addition of lactate or reducing agent ($Na_2S_2O_4$). On cooling the suspension with liquid air the band resolved into a double band. In some suspensions the double band was clearly visible at room temperature. This band is due to cytochrome b_1. (iv) A faint broad band at 530 mμ., presumably comprising the β bands of cytochromes a_2, a_1 and b_1.

When the suspension was treated with a drop of N-NaOH and a few crystals of $Na_2S_2O_4$, and pyridine was added to a final concentration of 20 per cent, the band at 630 mμ. disappeared, due to the destruction of cytochrome a_2 by alkali,

and the band at 560 mμ. was replaced by a broader and more intense band at 550–560 mμ. of pyridine haemochromogen. All the known haemochromogens, including cytochromes and pyridine haemochromogens, have approximately the same molecular extinction coefficients for their α bands. Keilin and Hartree (1949) and Slater (1949) therefore assume that the increase in absorption in the presence of pyridine and alkali is due to an increase in the absorption by haem derived, not from cytochrome, but from other haem proteins, such as catalase, peroxidase, etc., which have diffuse absorption bands that are not ordinarily visible. In this paper the haem convertible to pyridine haemochromogen is referred to as " total haem " without further enquiry into its origin.

A batch of cells grown in a medium containing 1 μg. Fe per ml. was extracted with acid-acetone according to the method of Rawlinson and Hale (1949) and the haems transferred through ether to 0·1 N-KOH. The haems in solution in acetone-HCl, ether, 0·1 N-KOH, and 0·1 N-KOH + $Na_2S_2O_4$ + pyridine had absorption bands in the same positions as similar solutions derived by Rawlinson and Hale from the cytochrome *b* of *C. diphtheriae* and from authentic protohaemin 9.

Estimation of Haems.

Bacterial haems were estimated in the comparison microspectroscope of Keilin (see Keilin and Hartree, 1946 ; Elliott and Keilin, 1934). In this device a powerful beam of light from a Pointolite lamp is passed through a colourimeter cup containing a thick suspension of bacteria into a low-dispersion microspectroscope ; at the same time another beam of light, the intensity of which can be varied with a rheostat, passes through a wedge-shaped glass trough containing a standard solution of pyridine haemochromogen and is then directed into the same microspectroscope. In this way the spectra of the light beams passing through the bacterial suspension and the standard can be seen side by side in the eyepiece of the spectroscope, and their intensities can be matched by manipulating the rheostat. The intensities of the absorption bands within these spectra can then be matched by manipulating the wedge containing the standard. From the depth of suspension in the colourimeter cup, the concentration of bacteria in the suspension, the concentration of the standard haemochromogen solution and the width of the wedge, the concentration of haems in the cells can be calculated. The standard solution of 0·0154 μMol. pyridine haemochromogen per ml. was prepared freshly every day by adding 10 ml. pyridine and 100 mg. $Na_2S_2O_4$ to 1 ml. of a stock solution of 0·77 μMol. recrystallised haemin per ml. 0·032 N-NaOH, and making up to 50 ml. with water.

To test whether a reliable estimate of bacterial cytochromes and haems could be obtained in a thick bacterial suspension scattering a great deal of light, the following experiment was done. A solution of pure cytochrome *c* was spectroscopically standardised in a Hilger Uvispek absorption spectrophotometer and two identical series of dilutions were made in phosphate buffer, pH 7·8. To one series of dilutions were added equal volumes of buffer, and to the other equal volumes of a suspension containing 80 mg. dry weight of washed *Clostridium welchii* cells per ml. (*Cl. welchii* contains no haem proteins). A few crystals of $Na_2S_2O_4$ were added to each dilution in both series and they were then matched against the standard pyridine haemochromogen solution in the comparison microspectroscope. With a little practice it was quite easy to match the narrow cytochrome *c* band at 550 mμ. with the broader pyridine haemochromogen band

at 550–560 mμ. The results of this experiment are shown in Fig. 2. It will be seen that a satisfactory estimation of cytochrome *c* was made at all dilutions and that light scattering by the thick bacterial suspension did not affect the results significantly.

In the experiments discussed below haem analyses were carried out on washed suspensions of *Sh. shigae* in the following way. Samples of bacterial suspension containing 40–60 mg. organisms per ml. phosphate buffer, pH 7·8, and varying in volume from 0·25 to 1 ml. according to their iron content, were pipetted into the colourimeter cup. A few crystals of $Na_2S_2O_4$ were added and the cytochrome b_1 band at 560 mμ. matched against the 550–560 mμ. band of the standard. If

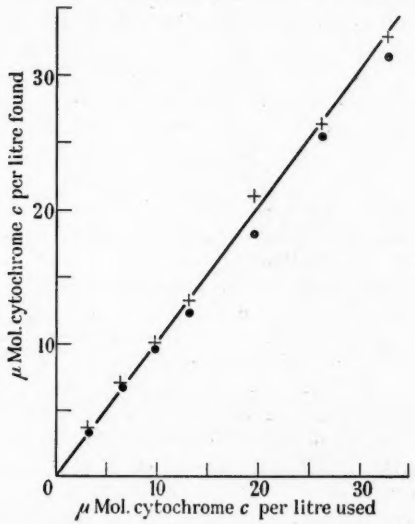


FIG. 2.—Determination of cytochrome *c* in simple solution (●) and in solution containing a suspension of *Cl. welchii* (+). The straight line represents theoretical values.

it is assumed that the molecular extinction coefficients of cytochrome b_1 and pyridine haemochromogen are the same the cytochrome b_1 haem content of the cells can be calculated from these data. Total haem was then estimated in the same samples of cells by adding a drop of N-NaOH and 0·1–0·3 ml. pyridine and thoroughly stirring the mixtures. The pyridine haemochromogen in the samples was then matched with that in the standard.

In all these experiments the intensity of the cytochrome b_1 band was between 50 and 60 per cent of that of the total haem in the same sample of organisms, and in the discussion below only the total haem content of the cells will be considered.

The Relation between Haem and Toxin Contents of Sh. shigae.

A series of 1 l. cultures of *Sh. shigae* were grown in 5 l. Mitchell flasks on successive days on media containing increasing amounts of iron and the toxin

and total haem contents of the organisms determined. The results are shown in curve A, Fig. 3. They show that the toxin content of the cells may increase as their haem content decreases, and are not inconsistent with a hypothesis on the lines suggested by Pappenheimer (1955). However, when the experiment was repeated some months later the totally different result shown in curve B, Fig. 3 was obtained. Here there is no consistent inverse relationship between toxin and haem. A number of further experiments were done in attempts to repeat the results shown in curve A, Fig. 3, but in all these cases it was found as the iron content increased up to about 0·2 μg. Fe per mg. organism, and the haem content increased correspondingly, the toxin content did not decrease, but remained constant, and since the growth yield increased the total toxin content of the culture increased. Only when the iron content of the cells was above about

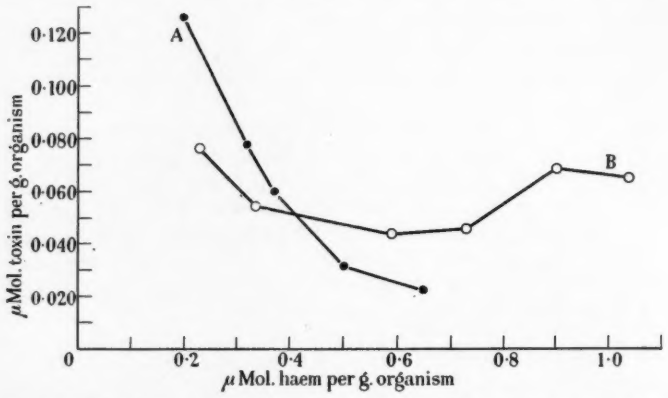


FIG. 3.—Relation between haem and toxin contents of *Sh. shigae* in two experiments.

0·2 μg. Fe per mg. did the toxin content decrease, and above this concentration of iron the haem content of the cells did not rise as sharply as it did at the lower concentrations. In earlier work (van Heyningen and Gladstone, 1953*b*) it was thought (despite, but not contrary to, the evidence of Fig. 4 in that paper) that toxin production invariably diminished with increasing iron content of medium. However, in the earlier work the medium was not as efficiently de-ferrated as in the present (see section on Methods) and, in addition, the values reported for the iron content of the earlier media were probably too low since only the iron adsorbable on calcium phosphate was determined.

Dependence of Toxin Production on Efficiency of Haem Synthesis.

Since the toxin content of the cells could not be shown to vary inversely with their haem content it seemed unlikely that the toxin might be related to the protein moiety of a bacterial haem protein. When the data that had hitherto been obtained were re-examined it became evident that the contrary may be true and that it is only when haem formation is inefficient that inhibition of toxin production takes place. This can be seen in Fig. 4, where iron, toxin and haem concentrations are plotted per unit volume of culture, rather than per unit weight

of cells, since all the iron is taken up by the organism. The broken lines passing through the origins in Fig. 4 represent quantitative conversion of medium iron to bacterial haems. It will be seen in all cases but the second that iron in low concentration is quantitatively converted into haem, and while this happens the total toxin content of the culture increases since the number of cells increases and their toxin content remains constant. In all cases including the second, when the conversion of iron to haem is incomplete, and the organisms thus contain some non-haem iron, there is inhibition of toxin production. Although low concentrations of iron appear generally to be converted into haems before being used in

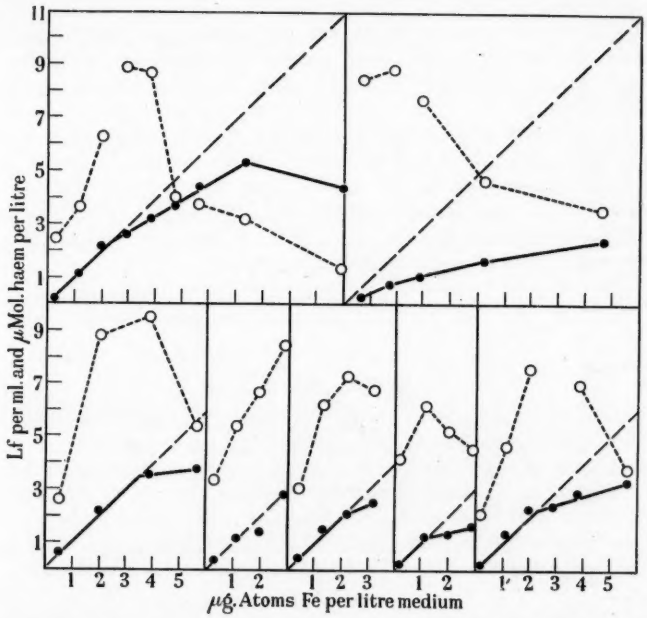


FIG. 4.—Relation between iron content of medium and toxin and haem contents of cultures of *Sh. shigae*. ○ = Lf per ml.; ● = μM haem per litre.

any other way, the second case in Fig. 4, which is the same as that shown in curve A, Fig. 3, shows that this is not always so.

Some inquiry has been made into the state of combination of the non-haem iron that inhibits toxin production. Organisms were grown in increasing concentrations of iron, and, in addition to determinations of growth yield, radioactive iron uptake, toxin and haem, estimations were made of the amount of iron in the cells that could be extracted with oxine-chloroform. The results of this experiment are shown in Fig. 5. As usual, the iron in the medium was taken up quantitatively by the organisms, low concentrations of iron were quantitatively converted into haem, and toxin production inhibited only when non-haem iron appeared in the cell. Haem iron in the cells does not react with oxine, but non-haem iron which inhibits toxin production is reactive. Up to a concentration of about 8 μg. atoms

Fe per litre all the iron can be accounted for as haem-iron alone, or as haem-iron plus oxine-reactive iron, but above this concentration iron appears in the organism in a third form which is neither haem nor oxine-reactive. It will be seen also that the concentration of iron at which toxin production is at its highest coincides with the point where no further increase in growth yield takes place.

The points representing toxin in Fig. 4 and 5 are joined together in groups of four or less since there were only four Mitchell flask whirlers available and consequently on any given day with a given inoculum not more than four cultures were put up. It will be seen that toxin production may vary considerably in cultures grown on exactly the same medium but inoculated at different times. Other

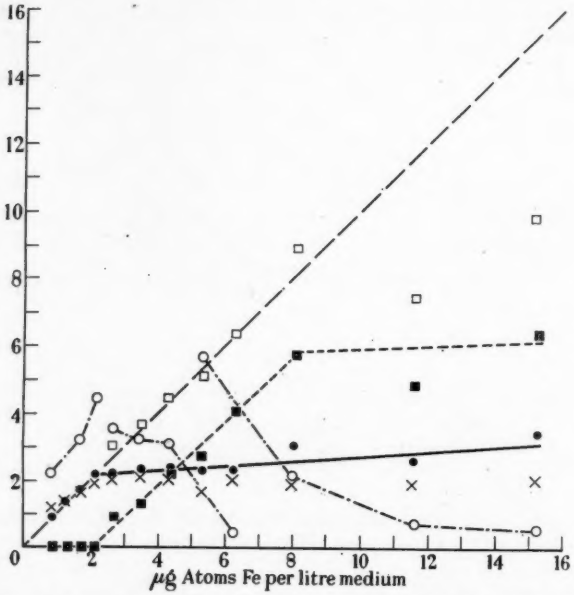


FIG. 5.—Relation between iron content of medium and growth yield, toxin content, haem content and oxine-reactive iron content of cultures of *Sh. shigae*. × = mg. organism per ml.; ○ = Lf per ml.; ● = μM haem per litre; ■ = μg. atoms oxine-reactive Fe per litre; □ = ● + ■.

factors besides the iron content of the organism, and the degree to which it is incorporated as haem, affect toxin production, since different yields of toxin are obtained in cells with the same content of oxine-reactive iron.

Whether or not the oxine-reactive iron can be extracted from the cells with chloroform appears to depend on whether the culture medium has been de-ferrated. The experiments on the extraction of iron with oxine-chloroform were done with cells grown on de-ferrated medium to which iron had been added. When non-toxic cells were grown in non-de-ferrated medium (containing about 3 μg. Fe per ml.) and treated with oxine-chloroform they became green-black, but the colour did not pass into the chloroform phase. On standing the green-black colour of the cells changed to the pink colour characteristic of ferrous-oxine.

Fig. 5 also shows that maximum toxin production takes place in cultures containing just enough iron to give the maximum growth yield. At lower concentrations of iron the concentration of toxin per cell is the same, but less cells and therefore less toxin are produced ; at higher iron concentrations less toxin is produced per cell, but the growth yield does not rise, and consequently toxin production falls. It is difficult, however, always to hit upon the optimal iron concentration and it would be useful to find conditions under which maximal growth is assured without inhibition of toxin production. Attempts at finding such conditions have been made.

(1) Since δ-amino-laevulic acid is known to be a precursor of porphyrins (Shemin and Russell, 1953) it was hoped that the addition of this substance to the medium would lead to a greater conversion of iron to haem. However, when cultures were grown in media containing from 0·5–1·0 μg. Fe per ml. and 20–100 μMol. δ-amino-laevulic acid per litre, no effect was observed on growth yield, haem synthesis or toxin production.

(2) It was hoped that if haematin were added to a de-ferrated medium instead of iron the organism would use it directly in the synthesis of haem proteins so that good growth yields could be obtained without an excess of iron to inhibit toxin production. Cultures were grown in the usual way in de-ferrated medium containing various amounts of haematin. It was found that haematin stimulated growth of the organisms, and haem formation in them, but inhibited toxin production. It appeared that the organism broke down the haematin before using the iron in it since a large proportion of it appeared in the cells in the oxine-reactive form.

DISCUSSION.

It is clear that toxin production in *Sh. shigae* is dependent on efficient haem formation, contrary to the original expectation that these two functions might be inversely related in the manner suggested by Pappenheimer in the case of diphtheria toxin. Although this finding explains the rôle of haem formation in the production of Shiga neurotoxin we still do not understand how iron exerts its inhibitory effect. It is possible that oxine-reactive iron in the cell inhibits protein synthesis generally, and that the effect of iron on toxin production is thus unspecific.

The production of toxin in *Sh. shigae* is controlled by a number of factors, only some of which are understood. In a given medium the growth yield will vary for unknown reasons with different inoculations. The greater the growth yield, the greater the total amount of toxin produced, not only because there are more cells to produce toxin, but also because these cells will contain less iron and therefore more toxin. In different batches of cells containing the same concentration of iron per cell, the efficiency of the conversion of iron to haem may vary for unknown reasons and this will affect the toxin content of the cells. In different batches of cells containing the same concentration of haem and of oxine-reactive iron the toxin content may still vary, for unknown reasons.

SUMMARY.

When *Sh. shigae* grows in a medium containing up to 1 μg. Fe per ml. all the iron is taken up by the organisms. At very low concentrations the iron is quantitatively taken up in haems ; at higher concentrations some of the iron is not converted

into haems, but is bound in the cells in a form that is reactive with 8-hydroxyquinoline ; in still higher concentrations iron is bound in the cells in a third form which is neither haem nor reactive with 8-hydroxyquinoline. As long as all the iron is converted into haems it does not affect toxin production, but when non-haem, oxine-reactive iron appears in the cell toxin production is inhibited.

There are other, unknown, factors besides the degree of conversion of iron to haem which affect toxin production.

Work on this toxin would be impossible without the dysentery antitoxin so generously and freely supplied by Miss Mollie Barr and Mrs. I. Batty of the Wellcome Research Laboratories, Beckenham. I am indebted further to Dr. R. J. P. Williams for helpful advice and discussion, and, as usual, I am grateful to Miss Winifred Crawford for highly competent technical assistance.

REFERENCES.

ELLIOTT, K. A. C. AND KEILIN, D.—(1934) *Proc. roy. Soc.*, B, **114**, 120.
GLADSTONE, G. P. AND FILDES, P.—(1940) *Brit. J. exp. Path.*, **21**, 161.
KEILIN, D. AND HARPLEY, C. H.—(1941) *Biochem. J.*, **35**, 688.
Idem AND HARTREE, E. F.—(1946) *Nature, Lond.*, **157**, 120.—(1949) *Biochem. J.*, **44**, 205.
MITCHELL, P.—(1949) *Nature, Lond.*, **164**, 846.
MOELLER, T.—(1943) *Industr. Engng Chem.* (*Anal.*), **15**, 346.
PAPPENHEIMER, A. M.—(1955) 'Mechanisms of Microbial Pathogenicity.' Ed. J. W. Howie and A. J. O'Hea. Cambridge (The University Press), p. 40.
RAWLINSON, W. A. AND HALE, J. H.—(1949) *Biochem. J.*, **45**, 247.
SHEMIN, D. AND RUSSELL, C. S.—(1953) *J. Amer. chem. Soc.*, **75**, 4873.
SLATER, E. C.—(1949) *Biochem. J.*, **45**, 1.
VAN HEYNINGEN, W. E. AND GLADSTONE, G. P.—(1953*a*) *Brit. J. exp. Path.*, **34**, 202.—(1953*b*) *Ibid.*, **34**, 221.
WOIWOD, A. J.—(1947) *Biochem. J.*, **41**, 39.

THE PRODUCTION OF BOTH INTERFERENCE AND ANTIBODY IMMUNITY BY PERTUSSIS VACCINE TO PERTUSSIS INFECTION IN MICE.

D. G. EVANS* AND F. T. PERKINS.*

From the Department of Bacteriology, University of Manchester.

Received for publication April 6, 1955.

WE previously showed (Evans and Perkins, 1954*b*) that a single intraperitoneal injection of pertussis vaccine produced in mice within a few hours a substantial immunity to an intracerebral injection of virulent *Haemophilus pertussis*. This effect, although specific for pertussis vaccine, appeared to be independent of circulating specific antibodies and it was suggested that the acquired immunity was probably the result of an interference effect, similar to that met with in the field of virology, rather than the production of antibody. Support for this suggestion was obtained from the results of further experiments (Evans and Perkins, 1954*c*) in which it was shown that pertussis vaccine given to mice intracerebrally at the site of infection, either with or three hours before the infecting dose of *H. pertussis*, was able to protect a high proportion of mice against infection. We have now shown that the early interference immunity following a single intraperitoneal injection of pertussis vaccine is of a transient type and that it is quite distinct from an immunity which develops more slowly and which manifests its full effect at a later stage when the interference immunity has faded. This slowly developing immunity is probably of a serological nature.

MATERIALS AND METHODS.

Vaccines.

Three pertussis vaccines, YPS, V16 and V17, were used. Vaccines YPS and V16 were whole bacterial vaccines each containing 20×10^9 killed organisms/ml. (U.S. opacity standard). YPS was the reference vaccine which we prepared for use throughout these studies (Evans and Perkins, 1954*a*). Vaccine V17 was a bacterial extract vaccine prepared from *H. pertussis* by sonic disintegration and contained only a small fraction of the whole bacillus absorbed on to human autoclaved red cell stromata. It was prepared by Pillemer, Blum and Lepow (1954) by the method they described.

Determination of active immunity in mice.

Female white mice of the Schofield strain were used ; this was a close-bred strain and has been used in all our previous studies. At the beginning of each test the average weight of the mice was 16 g. and the average age 4 weeks. Mice were injected intraperitoneally with 0·5 ml. of vaccine suitably diluted and the degree of immunity which developed at different times after injection determined by the intracerebral injection of a suspension of virulent *H. pertussis* (strain 18/323) according to the method described by Kendrick, Eldering, Dixon and Misner (1947). The challenge dose for these tests contained between 75 and 215 LD_{50} calculated by the method of Reed and Muench (1938) from the results of a control titration made with each test using 5 groups each of 15 mice. The average weight and age of the control mice were similar to those of the immunised mice at the time of challenge. Mice were observed for 21 days after challenge and the number of deaths recorded.

* Present address: National Institute for Medical Research, Mill Hill, London, N.W. 7.

Test for protective antibody in sera of immunised mice.

This test was made with the sera of mice immunised with pertussis vaccine. Sera were inactivated by heating at 56° for 30 min. In testing for small concentrations of protective antibody, 0·5 ml. of serum diluted 1/2 was injected intraperitoneally into groups of 10 mice which were challenged intracerebrally 18 to 20 hr. later with graded doses of a challenge suspension of *H. pertussis*. In testing sera containing higher concentrations of protective antibody, 0·5 ml. of graded dilutions of serum was given intraperitoneally to groups of 10 mice which were challenged 18 to 20 hr. later with a challenge dose containing about 100 LD_{50}. In each test the challenge dose was determined from the results of a control titration made with 5 groups each of 15 mice which, 18 to 20 hr. before challenge, were given an injection of normal mouse serum at the same dilution as the serum under test. The normal mouse serum used in all tests was the pooled sera from 200 mice. A control titration of the challenge dose was also made with mice which received no serum. Mice were observed for 21 days after challenge and the number of deaths recorded.

In vitro *tests for antibody in sera of immunised mice.*

Sera from mice immunised with pertussis vaccine were inactivated and titrated for agglutinin and complement-fixing antibody. The agglutination test was similar to that previously described (Evans and Perkins, 1953) in which a living suspension of *H. pertussis* was used as antigen. Two complement-fixation titrations were made each with a different antigen. One of the antigens was a whole bacterial suspension prepared by diluting vaccine V16 so that it contained $1{\cdot}5 \times 10^9$ organisms/ml. The other antigen was the bacterial extract vaccine V17 diluted 1/8. In both titrations 3 M.H.D. of complement and a 2 per cent suspension of sheep R.B.C. sensitised with 3 M.H.D. of haemolysin were used. Fixation was for 1 hr. at 37° and after adding the haemolytic system there was further incubation at 37° for ½ hr. In both the agglutination and complement-fixation titrations a control pertussis antiserum was always included with each batch of tests. Control tests were also made which showed that the stromata contained in the extract vaccine V17 played no part in the complement-fixation reaction.

Absorption of sera by whole bacteria.

A number of sera from immunised mice were absorbed with a living suspension of *H. pertussis* and titrated for agglutinin and complement-fixing antibody. In preparing the absorbing suspension, the 3-day growth of *H. pertussis* was scraped from plates of Bordet-Gengou medium and suspended in saline. This suspension was centrifuged and the packed deposit added to undiluted mouse serum; the growth from 20 plates of Bordet-Gengou medium was used for absorbing 1 ml. of serum. The mixtures of organisms and serum were incubated at 37° for 3 hr. before separating the absorbed serum.

EXPERIMENTAL AND RESULTS.

Duration of Immunity in Mice after a Single Intraperitoneal Injection of Vaccine.

In previous experiments (Evans and Perkins, 1954*b*), the development of immunity after a single injection of vaccine was followed for 10 days. During this period there was a rapid development of immunity which reached a high level 6 to 10 days after injection of vaccine. Experiments have now been made in which the immunity in mice was tested at various times up to 40 days after injection of vaccine. Three of the vaccines, YPS, V16 and V17 were used and a total of 12 different tests were made involving 1317 mice.

In each of these 12 tests the immunising dose of vaccine was 0·5 ml. of a 1/4 dilution. Groups of 10 to 15 mice were given vaccine at different times and all groups were challenged at the same time with an intracerebral dose of *H. pertussis*. The times at which the vaccine was given were arranged so that the groups of mice were challenged at times ranging from 5 hr. to 40 days after injection of vaccine.

TABLE I.—*Duration of Specific Immunity in Mice after a Single Dose of Pertussis Vaccine.*

Vaccine.	Number of tests.	Proportion (and percentage) of mice surviving challenge. Interval between immunising dose and challenge dose. 5 hr.	1 day.	3 days.	6 days.	10 days.	14 days.	20 days.	30 days.	40 days.	Challenge dose only.
V16	4	11/30 (37)	24/59 (41)	38/45 (84)	48/57 (84)	38/42 (91)	50/58 (86)	51/57 (90)	26/27 (96)	27/29 (93)	0/60 (0)
V17	6	14/55 (25)	31/85 (36)	42/58 (72)	71/81 (88)	64/67 (96)	56/75 (75)	76/80 (95)	26/28 (93)	24/25 (96)	0/90 (0)
YPS	2	9/15 (60)	18/30 (60)	9/15 (60)	27/30 (90)	28/28 (100)	24/29 (83)	13/13 (100)	19/19 (100)	—	0/30 (0)
Total	12	34/100 (34)	73/174 (42)	89/118 (76)	146/168 (87)	130/137 (95)	130/162 (80)	140/150 (93)	71/74 (96)	51/54 (95)	0/180 (0)

The immunising dose was 0·5 ml. of vaccine diluted 1/4.
The challenge doses contained between 75 and 215 LD_{50}.

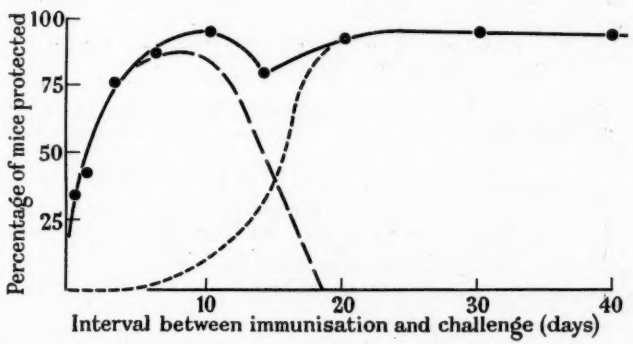


FIG. 1.—The development of immunity to pertussis infection in mice after a single injection of pertussis vaccine. Observed immunity ———; interference immunity — —; antibody immunity – – – –.

The results of these tests (Table I) confirmed our earlier observations by showing the rapid development of immunity following the injection of vaccine. With each vaccine at 5 hr. after injection a high proportion of mice were immune and at 6 to 10 days almost all the animals survived. The results also showed that almost all the mice survived when challenged 20 to 40 days after immunisation; the immunity during this period was just as good as that at 10 days. At 14 days after injection, however, there was some evidence of a decline in the level of immunity. This decline was not striking, but nevertheless the proportion of mice surviving at 14 days was, with each vaccine, always less than that at either 10 or 20 days. This decline is quite evident from Fig. 1 in which the results of all the tests are grouped together.

These results suggested that the immune response curve following a single intraperitoneal injection of vaccine was probably made up of two separate curves,

one due to an early interference and more transient type of immunity and a second due to the slower development of a true antibody type of immunity. These probable curves are indicated in Fig. 1. It was considered that if this hypothesis were true, then a more distinct separation of the two effects might be achieved by immunising with smaller doses of vaccine, and experiments along these lines are described in the next section.

Evidence of Two Distinct Immune Response Effects in Mice following a Single Intraperitoneal Injection of Vaccine.

In these experiments three of the vaccines YPS, V16 and V17, were used. With each vaccine 18 groups, each of 15 mice, were injected with 0·5 ml. of diluted vaccine; 6 groups were given vaccine diluted 1/4, another 6 groups vaccine diluted 1/10 and the remaining 6 groups vaccine diluted 1/25. At each immunising level, the groups were injected at different times and all groups were challenged at the same time with an intracerebral injection of 215 LD_{50}. The times at which the vaccine was given were so arranged that for each immunising dose the interval between the time of immunisation and challenge, for each of the 6 groups, was 1, 6, 9, 14, 20 and 30 days respectively.

TABLE II.—*Development of Two Immune Response Effects in Mice after a Single Dose of Pertussis Vaccine.*

Vaccine.	Mice immunised with 0·5 ml. of vaccine diluted:	Proportion (and percentage) of mice surviving when challenged at the following times after immunisation (days). 1.	6.	9.	14.	20.	30.
V16	1/4	5/15	10/14	14/14	14/14	14/15	15/15
	1/10	2/15	6/15	13/15	10/12	9/15	13/13
	1/25	1/15	2/15	11/14	5/15	7/15	12/15
V17	1/4	2/15	12/14	14/14	11/14	15/15	14/14
	1/10	2/15	7/15	12/14	9/15	12/15	12/12
	1/25	3/15	1/15	11/15	8/14	5/15	11/14
YPS	1/4	5/15	12/15	15/15	14/15	13/13	19/19
	1/10	1/15	14/15	12/14	7/14	11/14	13/13
	1/25	2/15	8/13	11/15	9/14	10/15	13/15
Total	1/4	12/45 (27)	34/43 (79)	43/43 (100)	39/43 (91)	42/43 (98)	48/48 (100)
	1/10	5/45 (11)	27/45 (60)	37/43 (86)	26/41 (63)	32/44 (73)	38/38 (100)
	1/25	6/45 (13)	11/43 (26)	33/44 (75)	22/43 (51)	22/45 (49)	36/44 (82)

The challenge dose contained 215 LD_{50}.

The results of these tests are shown in Table II and the combined results are plotted in Fig. 2. The combined results which are typical of those with each vaccine show that a decline in the degree of immunity occurred from 9 to 14 days after the injection of each immunising dose. This decline was only slight after immunisation with vaccine diluted 1/4, which gave a result similar to that shown in Fig. 1. It was most striking, however, when vaccine diluted 1/10 and 1/25 was used. After immunisation with a 1/10 dilution of vaccine, 86 per cent of mice

survived the challenge at 9 days and 63 per cent at 14 days, and after immunisation with vaccine diluted 1/25 the corresponding figures were 75 and 51 per cent. At 20 days after immunisation the degree of immunity, with each of these two immunising doses, was still low, but at 30 days it returned to a high level; 100 per cent of the mice immunised with vaccine diluted 1/10 were protected and 82 per cent of those immunised with vaccine diluted 1/25.

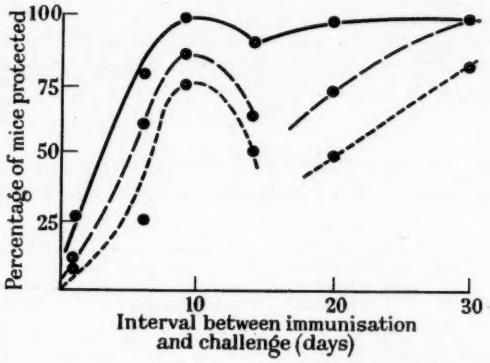


FIG. 2.—The production of two immune response effects in mice following a single injection of pertussis vaccine. Vaccine $\frac{1}{4}$ ———; vaccine $\frac{1}{10}$ — —; vaccine $\frac{1}{25}$ – – – –.

These results clearly indicated that each vaccine produced two immune response effects, an early rapid transient response and a later slower response which showed no evidence of waning at 30 days when the test was terminated. The suggestion that this later response was of the antibody type was supported by the results of titrations of specific antibody in the sera of immunised mice.

Production of Antibodies in Mice following a Single Intraperitoneal Injection of Vaccine.

Titrations were made of specific antibody in the sera of mice during a 42-day period after a single injection of vaccine. The tests were made with vaccines V16 and V17. For each vaccine seven groups of mice, 50 mice in each group, were given intraperitoneally 0·5 ml. of vaccine diluted 1/4. At various times, up to 42 days after injection, the groups were bled and the sera from each group pooled and inactivated. The pooled sera were titrated for agglutinin and complement-fixing antibody; in the latter test two different antigens, V16 and V17, were used. These pooled sera were also tested for protective antibody.

Agglutination and complement-fixation tests were also made with absorbed sera. For these tests, 3 additional groups each of 25 mice were immunised with vaccine V16 and another 3 similar groups with vaccine V17. With each vaccine the groups were bled at 21, 28 and 42 days after injection and the sera from each group pooled and inactivated. A small portion of each serum was absorbed and titrated for agglutinin and complement-fixing antibody in parallel with the unabsorbed sera.

In vitro *tests with unabsorbed sera.*

The results of these tests are shown in Table III. The sera from the mice immunised with V16 showed a gradual increase in agglutinin titre as the interval between the time of immunisation and bleeding increased; the highest titres of 40 to 160 were given by those sera taken 21 to 42 days after immunisation. Similarly with the complement-fixation titrations. In the tests in which V16 was used as antigen, no fixation was evident up to 14 days, but at 21 to 42 days some fixation occurred, although at only a low titre. In the tests using V17 as antigen, fixation was much stronger and a gradual increase in titre was observed as the interval between the time of immunisation and bleeding increased. The highest titres in these tests were 16 and 32 shown by sera taken at 30 and 42 days respectively.

TABLE III.—In vitro *Tests for Specific Antibody in Sera of Mice Immunised with a Single Dose of Pertussis Vaccine.*

Vaccine.	Number of days after inoculation when sera taken.	Antibody titres of sera.		
		Agglutinin.	Complement-fixing with antigen: V16.	Complement-fixing with antigen: V17.
V16 (whole bacteria)	3	<5	<2	<2
	5	5	<2	<2
	10	10	<2	2
	14	10	<2	4
	21	40	2	4
	30	80	2	16
	42	160	2	32
V17 (bacterial extract)	3	<5	<2	<2
	5	<5	<2	<2
	10	<5	<2	2
	14	<5	<2	8
	21	5	<2	64
	30	5	<2	64
	42	5	<2	64
Normal mouse serum		<5	<2	<2

The tests at each of the various times, with each vaccine, were made on the pooled sera from 50 mice immunised with 0·5 ml. of vaccine diluted 1/4.

The sera from the mice immunised with the extract vaccine V17 gave little or no antibody titres when tested against a whole bacterial suspension either in the agglutination or the complement fixation test. No agglutinin was detected up to 14 days and from 21 to 42 days a titre of only 5 was obtained, while in the complement fixation test using whole bacterial suspension as antigen, no fixation was detected throughout the whole period of 42 days. When the bacterial extract V17 was used as antigen in the complement fixation test, however, there was a gradual increase in titre which, from 21 to 42 days, was maintained at 64.

The result of these tests showed that those antibodies which could be determined by *in vitro* methods reached their highest level approximately 21 to 42 days after immunisation, but that during the earlier period they were either present at a lower level or not detectable. In fact the development of antibodies followed very closely the slowly developing immune response curve shown in Fig. 1 and 2 and which we have suggested was due to an immunity of the antibody type.

In vitro *tests with absorbed sera.*

The results of the *in vitro* titrations indicated that at least two different antigen-antibody reactions were involved, one taking part in the agglutination test and probably in the complement fixation test using whole bacteria as antigen and the other taking part in the complement fixation test using the bacterial extract. Tests were made with absorbed sera to determine whether these two reactions were separable. The results of these tests are shown in Table IV. With

TABLE IV.—In vitro *Tests for Specific Antibody in Absorbed and Unabsorbed Sera from Mice Immunised with a Single Dose of Pertussis Vaccine.*

Vaccine.	Number of days after inoculation when sera taken.	Unabsorbed (U) or absorbed (A).	Antibody titres of sera. Agglutinin.	Complement-fixing with antigen: V16.	Complement-fixing with antigen: V17.
V16 (whole bacteria)	21	U	80	2	8
		A	<5	<2	4–8
	28	U	160	2	8
		A	<5	<2	4–8
	42	U	160	2	16
		A	<5	<2	16
V17 (bacterial extract)	21	U	5	<2	64
		A	<5	<2	32–64
	28	U	5	<2	128
		A	<5	<2	128
	42	U	5	<2	64
		A	<5	<2	64

The tests at each of the various times, with each vaccine, were made on the pooled sera from 25 mice immunised with 0·5 ml. of vaccine diluted 1/4.

each serum, absorption with a whole bacterial suspension completely removed both the agglutinin and complement-fixing antibody against whole bacterial suspension but left almost unaltered the complement-fixing antibody against the bacterial extract. It is thus clear that a whole bacterial vaccine, such as V16, was able to produce on injection into mice at least two distinct antibodies, one to surface antigen and the other to deeper antigen. It is also clear that the bacterial extract vaccine contained only a small proportion of surface antigen, a result which agrees with previous observations (Evans and Perkins, 1955), but a high proportion of the deeper antigen. In fact, it is probable that a higher concentration of the deeper antigen was present in the extract vaccine V17 than in the whole bacterial vaccine V16, since higher complement-fixation titres, using extract as antigen, were invariably obtained with sera prepared against V17 than with those against V16.

Tests for protective antibody.

Previously we showed that sera taken from mice 3 or 9 days after injection of vaccine contained no detectable protective antibody when tested by an *in vivo* test. The results of tests (Table V) made over a 42-day period after immunisation

have now shown that protective antibody was not detectable in sera taken either during the early or late period after immunisation. Normal mice, which were given intraperitoneally 0·5 ml. of a 1/2 dilution of serum, taken from immunised mice at various times up to 42 days after injection of vaccine, were as susceptible to intracerebral infection as control mice which received either no serum or normal mouse serum.

TABLE V.—*Tests for Protective Antibody in Sera of Mice Immunised with a Single Dose of Pertussis Vaccine.*

Serum given 18–20 hr. before challenge.	Proportion of mice surviving after challenge with the following doses (LD_{50}).		
	500.	50.	10.
From mice injected with V16:			
5 days previously	—	0/10	—
10 „ „	—	0/10	—
14 „ „	0/10	0/10	1/10
21 „ „	0/10	0/10	3/10
30 „ „	0/10	0/10	0/10
42 „ „	0/10	0/10	3/9
From mice injected with V17:			
5 days previously	—	0/10	—
10 „ „	—	0/10	—
14 „ „	0/10	0/10	1/10
21 „ „	0/10	0/10	0/10
30 „ „	0/10	0/10	1/10
42 „ „	0/10	0/10	3/10
From normal mice	0/15	0/15	1/15
No serum	0/15	0/15	0/15

The dose of serum was 0·5 ml. of a 1/2 dilution.

It is not clear why protective antibody was not detected especially in those sera taken during the late period after immunisation. It is probable that a single injection of vaccine produced only a low level of circulating protective antibody which the test was incapable of detecting. In testing for protective antibody, serum was given intraperitoneally and by this route of injection may have been unable to produce a high enough concentration of antibody at the site of challenge to control the infectious process in the brain. That a basal level of protective antibody was laid down by a single injection of vaccine was evident from the results of tests made with sera from mice which received a second injection of vaccine.

Production of Antibodies in Mice after Two Spaced Injections of Vaccine.

Titrations were made of specific antibody in the sera of mice which received two spaced injections of pertussis vaccine. The tests were made with vaccines V16 and V17. For each vaccine 3 groups of 50 mice were injected with 0·5 ml. of vaccine diluted 1/4. One of the groups was given a second similar dose 14 days after the first and the mice bled 10 days later. The other two groups were given a second similar dose 28 days after the first and each group bled at 7 and 12 days after the second dose respectively. The sera from each of the 3 groups were pooled and inactivated. The pooled sera were titrated for agglutinin and complement-fixing

antibody, using in the latter test the two different antigens V16 and V17, and they were also tested for protective antibody.

The results of these tests are shown in Table VI and may be compared with those obtained after a single dose of vaccine (Table III). As was to be expected, the titres after two doses of vaccine were appreciably higher than those at any stage after one dose. The titres, however, followed the same pattern as before. Higher agglutinin and complement-fixing titres, using whole bacteria as antigen, were shown by the sera from mice immunised with V16 than by those from mice immunised with V17. Sera against both vaccines, however, gave good complement-fixing titres, using the bacterial extract as antigen, although the titres of the sera from mice immunised with V17 were higher than the corresponding titres of the sera from mice immunised with V16.

TABLE VI.—In vitro *and* in vivo *Tests for Specific Antibody in Sera of Mice Immunised with Two Spaced Doses of Pertussis Vaccine.*

Vaccine.	Number of days between the two immunising doses.	Number of days after second dose when sera taken.	*In vitro* antibody titres of sera. Agglutinin.	Complement-fixing with antigen: V16.	Complement-fixing with antigen: V17.	Protective antibody. Proportion of mice surviving challenge after receiving 0·5 ml. of serum diluted: 1/2.	1/4.	1/8.
V16 (whole bacteria)	14	10	1600	32	64	4/10	1/10	0/10
	28	7	3200	64	128	8/10	4/10	4/10
	28	12	6400	128	128	8/10	4/10	4/10
V17 (bacterial extract)	14	10	50	2	128	3/10	2/10	1/10
	28	7	100	2	256	8/10	2/10	2/10
	28	12	100	2	256	7/9	2/10	3/10
Normal mouse serum			<5	<2	<2	0/15	0/15	0/15

The challenge dose contained 128 LD_{50}.

The results of the tests for protective antibody, also given in Table VI, show that each serum had protective action. Especially was this evident with the sera taken from mice which received two doses of vaccine spaced at an interval of 28 days; 0·5 ml. of 1/2 dilution of sera from mice immunised with V16 protected 16 of 20 mice, and a similar dose of sera from mice immunised with V17 protected 15 of 19 mice, all of which were challenged with 128 LD_{50}. The protective property of the sera, however, was not high, since the effect was appreciably reduced when they were tested at either a 1/4 or 1/8 dilution.

It is thus evident that although protective antibody was not detected in serum after one injection of vaccine, some basal level must have been established at this stage since a second injection of vaccine produced a secondary response of protective antibody. It is highly probable that this basal level of protective antibody was responsible for the slower developing active immunity (Fig. 1 and 2), which occurred after a single injection of vaccine and which reached a high level between 20 and 40 days, and not with the early rapid transient response. This view is supported by the fact that when the second injection of vaccine was given 28 days after the first, the serum had an appreciably greater protective effect than serum resulting from two injections separated by an interval of 14 days.

DISCUSSION.

In a recent *World Health Organisation Report* (1953) it was considered that the mouse protection test, which has been used extensively for a number of years for testing the prophylactic potency of pertussis vaccines, was sufficiently well established to justify its employment for the purpose of routine control. The test necessitates the immunisation of mice with a single intraperitoneal injection of vaccine and the determination of the degree of immunity, 14 days later, by the intracerebral challenge injection of virulent *H. pertussis*. This test, in view of the results of our experiments, must be given careful reconsideration. The results indicate that when mice are challenged 14 days after injection of vaccine, a determination is being made of the ability of the vaccine to produce an interference immunity and not an antibody immunity. At 14 days, the antibody immunity is not fully developed and it is only at a later stage, probably at about 30 days after injection of vaccine, that the antibody immunity reaches its highest stable level. Thus if the test is to be a true measure of the ability of a vaccine to produce antibody immunity, the challenge injection should be given about 30 days after immunisation.

It may, of course, be argued that the antigen responsible for producing the antibody immunity is also the responsible agent in the production of the early interference phenomenon and that the resistance which develops during the early period after injection of vaccine is an indication of the immunity which develops later. There is at present no proof of this ; but even though the same entity were involved in the production of both the late and early immunity, 14 days after injection of vaccine is, nevertheless, an unsuitable time at which to determine the degree of immunity. At 14 days a most unstable immune state exists ; the early interference effect is rapidly waning and the antibody immunity is slowly building up. In fact, immunity at this stage is probably in a more unstable state than at any other time throughout the whole period of 40 days after injection of vaccine. It is, therefore, not surprising that the inaccuracies of the test are such that a high degree of precision can only be achieved by the use of an inordinately large number of mice (*World Health Organisation Report*, 1953).

It is not yet known whether the antibody responsible for the protection of mice against intracerebral pertussis infection is associated with any of the antibodies measurable by *in vitro* methods. Antisera with protective activity have been shown by *in vitro* tests to contain at least two distinct antibodies. One takes part in agglutination with whole bacilli and is presumably an antibody to surface antigen. The other fixes complement in the presence of a bacterial extract and is an antibody to deeper antigen, since it does not fix complement in the presence of whole bacilli and is not absorbed by whole bacilli. We have considered that antibody to surface antigen may be of significance in protection against pertussis infection and have suggested that the ability of a vaccine to produce agglutinin may be an indication of its protective action (Evans and Perkins, 1953, 1954*a*). The bacterial extract vaccine prepared by Pillemer *et al.* (1954), however, produces antisera with low agglutinin titres (Evans and Perkins, 1955) and yet protects mice just as effectively as whole bacterial vaccines which produce relatively high agglutinin titres. It is, of course, possible that more than one surface antigen is involved in the agglutination test and that the bacterial extract vaccine contains the one which plays the most important part in protection. Some support for this view has already been obtained from a few preliminary experiments using antisera

prepared in rabbits by immunisation with bacterial extract. It was shown that these antisera had high protective property, contained both agglutinin (titre 800) and complement-fixing antibody to the deeper antigen (titre 512), and that when absorbed with whole bacilli were hardly affected in their ability to fix complement but completely lost both their agglutinin and protective property. Further investigations along similar lines are now being made.

SUMMARY.

It has been shown that two distinct immune response effects are produced in mice after a single intraperitoneal injection of pertussis vaccine. The first is of an interference type ; it is transient, it occurs rapidly, reaches its maximum effect in about 10 days and then quickly fades. The second is of an antibody type ; it occurs slowly, reaches a high level in about 20 to 40 days when it shows no evidence of waning. It has also been shown that the slow immune response occurs in parallel with the production of agglutinin to surface antigen and of complement-fixing antibody to deeper antigen. Although protective antibody is not detected in sera after one injection of vaccine it is evident that a basal level is established at this stage, since a second injection of vaccine produces a secondary response of protective antibody.

It is suggested that in testing the prophylactic potency of pertussis vaccines by the mouse protection test, the degree of immunity should be determined at about 30 days after injection of vaccine and not at 14 days when a most unstable immune state exists.

We wish to thank the Medical Research Council for samples of pertussis vaccine received from the Whooping-Cough Immunisation Committee and for a grant towards the expenses of this work.

REFERENCES.

Evans, D. G. and Perkins, F. T.—(1953) *J. Path. Bact.*, **66**, 479.—(1954*a*) *Ibid.*, **68**, 251.—(1954*b*) *Brit. J. exp. Path.*, **35**, 322.—(1954*c*) *Ibid.*, **35**, 603.—(1955) *J. Path. Bact.*, **69**, 329.

Kendrick, P. L., Eldering, G., Dixon, M. K. and Misner, J.—(1947) *Amer. J. publ. Hlth*, **37**, 803.

Pillemer, L., Blum, L. and Lepow, J. H.—(1954) *Lancet*, i, 1257.

Reed, L. J. and Muench, H.—(1938) *Amer. J. Hyg.*, **27**, 493.

World Health Organisation Report—(1953) *Tech. Rep. Wld Hlth Org.*, No. 61.

NATURALLY OCCURRING PROTEIN DROPLETS IN THE PROXIMAL TUBULE OF THE RAT'S KIDNEY.

J. LOGOTHETOPOULOS AND K. WEINBREN.

From the Pathology Department, Postgraduate Medical School of London, London, W.12.

Received for publication April 7, 1955.

INTRACELLULAR hyaline droplets in the proximal tubules of the kidney have been described after the injection of protein solutions in the toad (Gerard, 1935), salamander (Lambert, 1936) and in mammalian species (Smetana, 1947). Since then these inclusions have been analysed by different techniques and the general view is that they represent protein material re-absorbed from the glomerular filtrate (Oliver, 1948; Rather, 1948). The more recent studies in this field have been concerned with the structural and functional relationship between these inclusions and cell organelles by phase contrast microscopy (Zollinger, 1950; Rüttimann, 1951), histochemical procedures (Oliver, Moses, MacDowell and Lee, 1954) and cell fractionation by differential ultracentrifugation and enzymic determinations (Kretchmer and Dickerman, 1954). In all the published reports the droplets studied were seen after intraperitoneal injections of mostly low-molecular proteins, although occasional reference is made to the occurrence of droplets not specifically induced by protein injections.

During preliminary attempts to induce droplets by injection of egg albumen in rats we noted the presence of large numbers of naturally occurring droplets in mature male controls but not in mature females. This observation, together with the known difference of proteinuria in the male and female rat (Bell, 1933) and the effect of androgens on the spontaneous proteinuria of rats (Sellers, Goodman, Marmorston and Smith, 1950) led us to attempt to record the occurrence of naturally occurring droplets, to find whether there was a difference in the two sexes and, if so, to see whether this difference could be correlated with the degree of proteinuria.

MATERIAL AND METHODS.

Rats.

Male and female albino rats were kept on a diet consisting of "Research" rat cubes supplemented with white bread and greens twice a week.

Protein estimation in urine.

At 8 to 9 a.m. food was withheld and the rats were allowed free access to a 10 per cent glucose in 0·5 per cent saline solution. Four hr. later they were individually placed in urine-collecting glass cages for a period of 7 to 8 hr. The collecting cages consisted of inverted 5-l. glass jars whose bases had been cut off and in which a wide-mesh wire floor supported the animal 2 in. above the neck of the bottle. Urine was collected through a glass funnel into a covered rubber-topped specimen jar after passing through a narrow-mesh wire faeces trap. All glassware was siliconed. During the period of collection the animals were allowed

10–15 ml. of the glucose solution in two doses. Emptying of the bladder was stimulated at the beginning and end of the collecting period by forced inhalation of ether.

Protein was estimated by the Biuret method (Foster, Rick and Wolfson, 1952), and a solution of crystalline bovine albumen was used as a standard.

Protein excretion was expressed as μg./100 cm.^{2} body surface/hr. Meeh's formula was used for deriving body surface from body weight ($S = KW^{2/3}$ with $K = 11{\cdot}36$) as determined by Carman and Mitchell (1926).

Microscopical preparations.

The kidneys were removed under ether anaesthesia, care being taken to avoid undue congestion during removal. They were weighed, longitudinally bisected and placed immediately in Helly's fluid. The left kidney was used for paraffin sections stained by H.E., Heidenhain's haematoxylin, phosphotungstic acid haematoxylin (P.T.A.H.), Bensley's modification of Altmann's anilin acid fuchsin, 0·5 per cent phloxine in 20 per cent alcohol using alkali alcohol differentiator and Ehrlich's haemalum nuclear stain, and Weigert's method for fibrin. Phase contrast microscopy and supravital preparation with Janus Green B were used on fresh material in selected cases. Pyridine extraction followed by Baker's acid haematin or Sudan black was used on frozen and paraffin sections.

Urine collection from the bladder.

This was carried out after injection of 0·06 mg. nembutal/100 g. body wt. and 2 ml. of 3 per cent ethanol in water/100 g. body wt. given by gastric intubation. After 1 hr. of anaesthesia, urine was collected by a suprapubic incision through a 22 gauge needle attached to a syringe.

RESULTS.

Normal rats.

Proteinuria was determined in 10 male and 10 female rats every two weeks from the ages of 30 to 120 days. Batches of 10 males and 10 females were killed every two weeks. An increase in protein excretion was found in male rats from 30 days onwards. A maximum of 180 μg./100 cm.^{2} body surface/hr. was reached by 90 days, approximately 2½–3 times that of female rats of the same age in whom protein excretion, apart from minor fluctuations, was relatively constant and did not show the marked elevation at puberty (Figure). This difference in protein content in the urine was reflected also in urine withdrawn by bladder puncture in 10 male and 10 female rats 4 months old. The value was 201 μg. $\pm$ S.D. 35/100 cm.^{2} body surface/hr. for the male group and 71 μg. $\pm$ S.D. 25/100 cm.^{2} body surface/hr. for the female group.

Intracellular droplets mainly in the upper two-thirds of the proximal tubules were found in male rats from 60 days onwards, the earliest arranged in small groups of small droplets in occasional cells. These increased in number and size at 75 days and were found in most of the cells in the proximal tubule at 90 days. The droplets were phloxinophilic, stained with P.T.A.H., Weigert's fibrin stain, Heidenhain's haematoxylin and supravitally with Janus Green B and were prominent under the phase contrast microscope. Mitochondria did not appear to be notably deficient in cells containing droplets in the single sections studied (see Plate). After pyridine extraction the droplets were not stained by acid haematin or Sudan black, but these reactions were positive before pyridine extraction. In contrast, only occasional female rats, up to 5 in a group of 20 at 120 and 180 days showed the presence of occasional droplets in a few cells of the proximal tubules.

Thus at puberty in male rats there is a well marked increase in the degree of proteinuria and, at the same time, abundant intracellular droplets are noted in

the proximal tubules of the nephron. Females show neither a rise in proteinuria nor intracellular droplets.

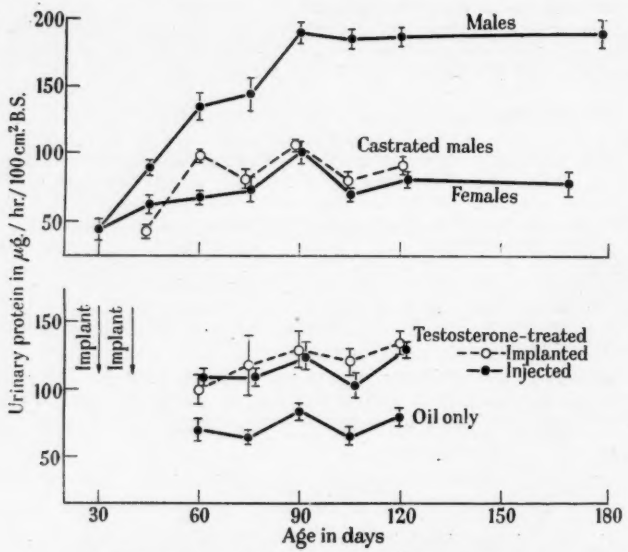


FIGURE.—Proteinuria in male and female rats.
Upper graph—males and castrated males.
Lower graph—testosterone-treated females.

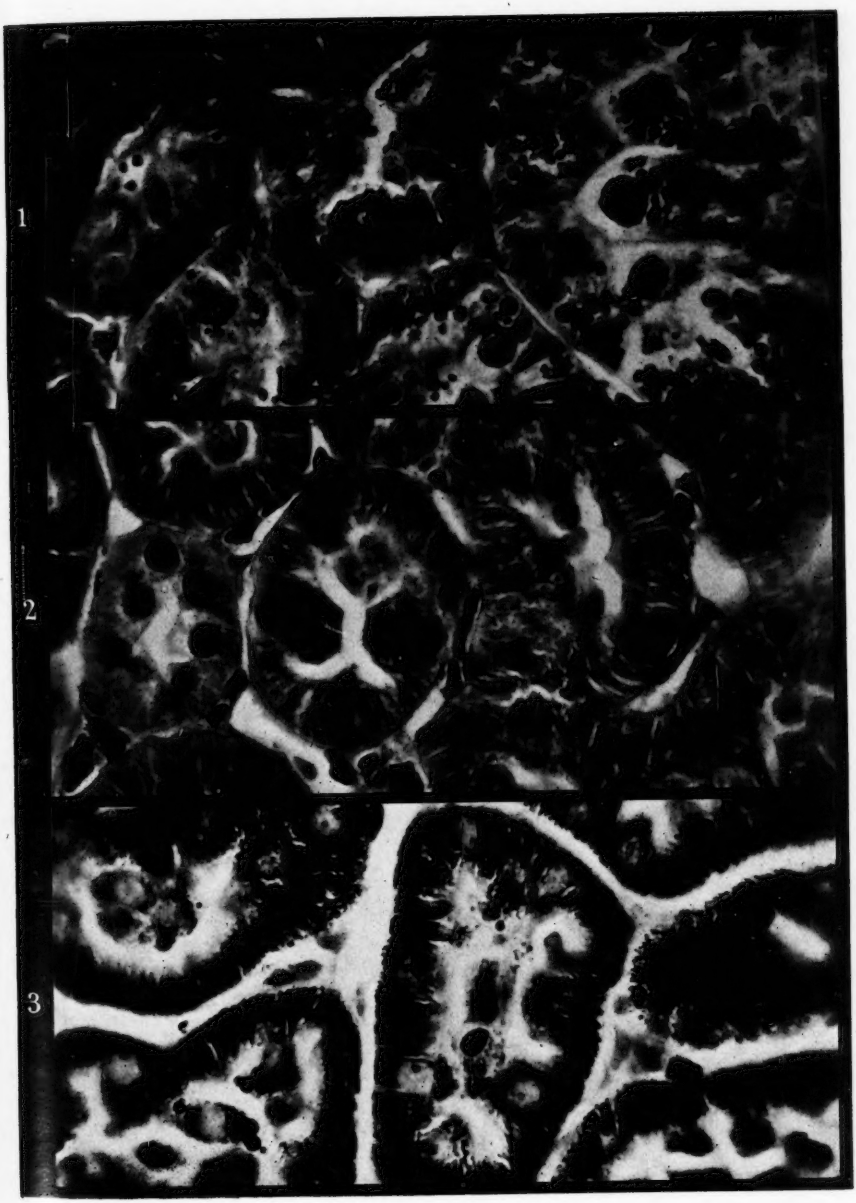
Castrated male rats.

Twenty male rats were castrated at 30 days of age and 20 litter mates kept as controls. Urinary protein excretion was measured every 15 days and the rats were killed and kidneys removed and examined in the usual way at 105 and 120 days. The amount of protein in the urine was significantly lower than that of normal male rats and was approximately equal to that found in females. The kidney weights were 1·45 g. ± S.D. 0·2 as compared with 1·89 g. ± S.D. 0·10 in the litter mate controls, body weights being similar in the two groups. The proximal tubule cells appeared smaller in the castrated groups. Moderate numbers of inclusions were found in only 4 castrated male rats. All the controls contained abundant droplets.

Proteinuria in castrated rats is significantly reduced and the incidence of hyaline inclusions is much reduced.

EXPLANATION OF PLATE.

1.—Section of kidney of adult male rat; phloxine and Meyer's haemalum: shows abundant cytoplasmic inclusions (× 800).
2.—Section of kidney of 75 day-old male rat; phloxine and Meyer's haemalum: shows inclusions in mitosing tubule cells (× 800).
3.—Section of kidney of adult male rat; Bensley's modification of Altmann's aniline acid fuchsin: shows abundant mitochondria and inclusions in proximal tubules (× 800).



Logothetopoulos and Weinbren.

Female rats treated with testosterone propionate.

Twelve female rats were injected subcutaneously with 5 mg. testosterone propionate in oil every second day from 50 days of age. Twelve control rats were given an equal volume of sterile almond oil. In a further 12 rats 25 mg. pellets of testosterone propionate (Organon) were implanted at 35 and 75 days. Proteinuria was measured in the usual way and the animals were killed and examined at 120 days of age. The protein excretion of animals receiving testosterone propionate was significantly higher than that of the controls (approximately 120 μg./100 cm.^{2} body surface/hr. compared with 60 μg./100 cm.^{2}/hr.). In six of the testosterone-treated female rats the level was greater than the average. Aerobic and anaerobic cultures were made of the kidney. No organisms were recovered and no microscopical evidence of renal inflammation was found.

The kidneys of the testosterone-treated rats weighed 1·84 g. $\pm$ S.D. 0·16 for the implanted, 1·85 g. $\pm$ S.D. 0·14 for the injected, compared with 1·30 g. $\pm$ S.D. 0·17 for the controls. No increase in the number of droplets was found in the experimental as compared with the control animals.

After treatment with testosterone propionate the protein excretion of female rats is increased to levels about mid-way between those of normal females and normal males, but the incidence of inclusions is not raised.

DISCUSSION.

The intrinsic difficulty in determining the relationship of these inclusions to protein re-absorption is the fact that we cannot obtain knowledge of the protein load of the glomerular filtrate. From this information and the amount of protein in the urine, an estimate of re-absorbed protein could be derived. An increased protein load at puberty in the male rat or a change in metabolism of the proximal tubule cells are both valid as interpretations of the present findings and similar arguments may be applied to the differences following castration.

The naturally occurring inclusions appear to conform exactly in morphology and staining reactions to those described after injections of foreign protein (Oliver *et al.*, 1954), but we were not able to demonstrate an inverse relationship between numbers of mitochondria and numbers of inclusions in any particular cell or group of cells.

The serial studies on cellular behaviour following the injection of low molecular proteins are subject to experimental error, as it seems possible that induced droplets may be confused with those occurring naturally. The conclusions may be rendered more valid if attention is directed to the evolution of naturally occurring inclusions in the species used and to the age and sex of the animals.

SUMMARY.

Abundant naturally occurring protein inclusions were found in the proximal tubules of male rats 60 days and older but not in any substantial numbers in female rats. These inclusions have been correlated with the level of proteinuria.

The effect of castration in male rats was to reduce the incidence of inclusions and to lower the level of proteinuria.

The effect of testosterone propionate in females was to raise the level of proteinuria. There was no increase in the content of inclusions.

This investigation was completed during the tenure of a British Council Scholarship by one of us (J. L.). We thank Messrs. J. G. Griffin for the sections and V. E. Willmott for the photographs.

REFERENCES.

BELL, MURIEL E.—(1933) *J. Physiol.*, **79**, 191.
CARMAN, G. G. AND MITCHELL, H. H.—(1926) *Amer. J. Physiol.*, **76**, 380.
FOSTER, PHYLLIS W., RICK, J. J. AND WOLFSON, W. Q.—(1952) *J. Lab. clin. Med.*, **39**, 618.
GERARD, P.—(1935) *J. Anat., Lond.*, **70**, 354.
KRETCHMER, N. AND DICKERMAN, H. W.—(1954) *J. exp. Med.*, **99**, 629.
LAMBERT, P. P.—(1936) *Arch. Biol., Paris*, **47**, 125.
OLIVER, J.—(1948) *J. Mt Sinai Hosp.*, **15**, 175.
Idem, MOSES, M. J., MACDOWELL, MURIEL C. AND LEE, Y. C.—(1954) *J. exp. Med.*, **99**, 605.
RATHER, L. J.—(1948) *Stanf. med. Bull.*, **6**, 117.
RÜTTIMANN, A.—(1951) *Schweiz. Z. allg. Path.*, **14**, 373.
SELLERS, A. L., GOODMAN, H. C., MARMORSTON, J. AND SMITH, MARGARET—(1950) *Amer. J. Physiol.*, **163**, 662.
SMETANA, H.—(1947) *Amer. J. Path.*, **23**, 255.
ZOLLINGER, H. U.—(1950) *Schweiz. Z. allg. Path.*, **13**, 146.

QUANTITATIVE STUDIES IN DIPHTHERIA PROPHYLAXIS: THE DETERMINATION OF THE ANTIGENICITY CHARACTERISTICS OF PURIFIED DIPHTHERIA TOXOID IN SIMPLE SOLUTION AND AS P.T.A.P.

L. B. HOLT AND JOAN M. BARNES

From the Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, W.2.

Received for publication April 7, 1955.

IT is well known that adsorbed toxoids such as A.P.T., P.T.A.P. or P.T.A.H. are much more powerful antigens than their parent fluid toxoids. If, however, one attempts to make a quantitative assessment of the *comparative* antigenicity of fluid toxoid and an adsorbed toxoid a number of difficulties arise. The major difficulties are that the dose-response lines (log geometric mean titre on log dose) may not be parallel and, if so, no simple comparison is possible, and that even if they were parallel a strict comparison could only be made provided the standard deviations were similar in the two population series.

An attempt has been made to overcome these difficulties by devising an antigenicity equation which contains, implicitly or explicitly, all the variables, and where no comparisons with a reference or standard preparation are involved (Holt, 1953 ; 1955).

The antigenicity equation described is

$$\log G.M._{(D)} = \log At_{(d)} + \sigma\, b \log \frac{D}{d}.$$

where

$\log G.M._{(D)}$ = geometric mean titre of response resulting from a dose D.

$\log At_{(d)}$ = *geometric mean* titre At, resulting from another dose d.

σ = standard deviation of logs of titres (supposed constant for all doses).

b = the slope of the regression line relating the probit of the percentage (of a group) exceeding the antitoxin *level* At to log dose (administered to the group).

The relationship between geometric mean titre and the probit of this percentage is

$$\log G.M. = \log At + \sigma\,(\text{Probit } y\% - 5).$$

It should be pointed out that the product of σ and b equals B the slope of the dose-response line relating log geometric mean titre to log dose.

A simplified formal proof of the antigenicity equation and of the relation between B and $\sigma\, b$ is given in the Appendix to this paper.

It will be noted that the antigenicity equation contains three variables: the median effective dose d, *i.e.*, the smaller this dose the better the prophylactic; σ, which gives a measure of the variability (scatter) of the responses in the population inoculated, *i.e.*, with a small σ we may expect a smaller percentage of gross failures for a given geometric mean titre; and b, this gives the degree of improvement in the percentage that exceeds some reference point of response to increase in dose, *i.e.*, when b is large a large improvement in proportionate success can be expected for a small increase in inoculum.

If, as contended by Prigge (1953; 1954), all dose-response lines are parallel and the slope B is always unity then it would be possible to express the antigenicity of one type of diphtheria prophylactic in terms of another by simple volume or Lf ratio and hence potency in terms of units of an antigenic preparation would be acceptable for all practical purposes.

The following experiments were done to determine the antigenicity values of purified diphtheria toxoid in simple solution and as P.T.A.P., a most important point being the measurement of B for the two forms of diphtheria prophylactic.

Some data have already been given on the estimation of these antigenicity values for P.T.A.P. (Holt, 1955). These present observations are more satisfactory in that the time interval between inoculation and bleeding has been doubled, more animals were used for each dose-level, and the response of each animal was measured and the geometric mean and σ calculated with adequate precision.

Groups of 25 guinea-pigs (about 300 g.) were used for each dose; the dose-volume was uniform at 1·0 ml. and the inoculations were made subcutaneously on the ventral wall. The time interval between inoculation and bleeding was 8 weeks; all guinea-pigs employed for one experiment were inoculated within a time interval of 20 days; a dose-ratio increment of fivefold was used and all sera were titrated, where possible, to an accuracy of $\pm$ 10 per cent and verified once using a method based on that of Römer and Sames (1909). The animals were all from the same inbred stock used in these laboratories for the last 15 years. Dose-response data were obtained for two samples of P.T.A.P. and for one sample of purified toxoid in simple solution; two dose-levels using the same sample of purified toxoid were repeated at a later date. The results obtained are shown in detail in Tables I and II and in summary in Fig. 1.

The median value of the group for the lowest dose of P.T.A.P. has been used in both cases as the geometric mean could not be calculated; even so the slope B for P.T.A.P. in guinea-pigs is clearly very different from that for F.T. Despite the fact that the *dose* required for a given geometric mean titre in Exp. 1 is greater than in Exp. 2 the two dose-response lines are substantially parallel. It should be recorded that the results (titres) obtained in Exp. 2 are more in keeping with our routine assay results than those from Exp. 1. The estimate of B for P.T.A.P. is 1·6 and for F.T. 0·61. Also it will be noted that the highest dose of P.T.A.P. (1 mg. $AlPO_4$ with 5 Lf toxoid) in Exp. 2 showed a falling-off effect. Indeed this was known, from previous dose-response work, to occur at about this level of dosage (Holt, 1950) and was the reason why greater quantities of P.T.A.P. were not used in this investigation. This falling-off effect has its counterpart at the other extreme (not recorded here in detail) where the animals, inoculated with either P.T.A.P. or F.T., divided themselves into those which make no measurable response and those which do (see also Barr and Llewellyn-Jones, 1951). We see,

TABLE I.—*Orderly Array of Individual Responses to Different Doses of P.T.A.P. in Guinea-pigs.*

	Exp. 1						Exp. 2							
	Group 1 (0·2 Lf).		Group 2 (1 Lf).		Group 3 (5 Lf).		Group 1 (0·04 Lf).		Group 2 (0·2 Lf).		Group 3 (1 Lf).		Group 4 (5 Lf)	
Guinea-pig Number.	Titre.	Log. titre.	Titre.	Log. titre.	Titre.	Log. titre.	Titre.	Log. titre.	Titre.	Log. titre.	Titre.	Log. titre.	Titre.	Log. titre.
1	<0·0005	<$\bar{4}$·700	Died		0·2	$\bar{1}$·3010	<0·0005	<$\bar{4}$·7	0·0005	$\bar{4}$·6990	0·06	$\bar{2}$·7781	0·125	$\bar{1}$·0960
2	<0·0005	<$\bar{4}$·700	0·001	$\bar{3}$·0000	0·35	$\bar{1}$·5441	<0·0005	<$\bar{4}$·7	0·0005	$\bar{4}$·6990	0·08	$\bar{2}$·9031	0·3	$\bar{1}$·4771
3	<0·0005	<$\bar{4}$·700	0·0015	$\bar{3}$·1761	0·35	$\bar{1}$·5441	<0·0005	<$\bar{4}$·7	0·0005	$\bar{4}$·6990	0·08	$\bar{2}$·9031	0·4	$\bar{1}$·6020
4	<0·0005	<$\bar{4}$·700	0·0025	$\bar{3}$·3979	0·4	$\bar{1}$·6020	0·0005	$\bar{4}$·6990	0·0005	$\bar{4}$·6990	0·09	$\bar{2}$·9542	0·4	$\bar{1}$·6020
5	<0·0005	<$\bar{4}$·700	0·006	$\bar{3}$·7782	0·4	$\bar{1}$·6020	0·0005	$\bar{4}$·6990	0·004	$\bar{3}$·6020	0·13	$\bar{1}$·1139	0·4	$\bar{1}$·6020
6	0·0025	$\bar{3}$·3979	0·006	$\bar{3}$·7782	0·5	$\bar{1}$·6990	0·0015	$\bar{3}$·1761	0·004	$\bar{3}$·6020	0·17	$\bar{1}$·2304	0·4	$\bar{1}$·6020
7	0·0025	$\bar{3}$·3979	0·04	$\bar{2}$·6021	0·5	$\bar{1}$·6990	0·0015	$\bar{3}$·1761	0·005	$\bar{3}$·6990	0·18	$\bar{1}$·2552	0·7	$\bar{1}$·8541
8	0·0025	$\bar{3}$·3979	0·05	$\bar{2}$·6990	0·5	$\bar{1}$·6990	0·0015	$\bar{3}$·1761	0·005	$\bar{3}$·6990	0·2	$\bar{1}$·3010	0·8	$\bar{1}$·9031
9	0·003	$\bar{3}$·4771	0·06	$\bar{2}$·7782	0·6	$\bar{1}$·7782	0·0015	$\bar{3}$·1761	0·005	$\bar{3}$·6990	0·2	$\bar{1}$·3010	0·8	$\bar{1}$·9031
10	0·004	$\bar{3}$·6020	0·06	$\bar{2}$·7782	0·6	$\bar{1}$·7782	0·0015	$\bar{3}$·1761	0·008	$\bar{3}$·9031	0·2	$\bar{1}$·3010	1·1	0·0414
11	0·004	$\bar{3}$·6020	0·08	$\bar{2}$·9031	0·8	$\bar{1}$·9031	0·0015	$\bar{3}$·1761	0·008	$\bar{3}$·9031	0·22	$\bar{1}$·3424	1·25	0·0969
12	0·004	$\bar{3}$·6020	0·10	$\bar{1}$·0000	0·9	$\bar{1}$·9542	0·0015	$\bar{3}$·1761	0·01	$\bar{2}$·0000	0·22	$\bar{1}$·3424	1·3	0·1139
13	0·005	$\bar{3}$·6990	0·125	$\bar{1}$·0969	1·1	0·0414	0·0015	$\bar{3}$·1761	0·01	$\bar{2}$·0000	0·25	$\bar{1}$·3979	1·3	0·1139
14	0·005	$\bar{3}$·6990	0·125	$\bar{1}$·0969	1·25	0·0969	0·002	$\bar{3}$·3010	0·015	$\bar{2}$·1761	0·3	$\bar{1}$·4771	1·5	0·1761
15	0·005	$\bar{3}$·6990	0·18	$\bar{1}$·2553	1·25	0·0969	0·004	$\bar{3}$·6020	0·04	$\bar{2}$·6020	0·35	$\bar{1}$·5441	1·7	0·2304
16	0·006	$\bar{3}$·7782	0·2	$\bar{1}$·3010	1·25	0·0969	0·004	$\bar{3}$·6020	0·05	$\bar{2}$·6990	0·4	$\bar{1}$·6020	1·7	0·2304
17	0·006	$\bar{3}$·7782	0·22	$\bar{1}$·3424	1·25	0·0969	0·004	$\bar{3}$·6020	0·05	$\bar{2}$·6990	0·5	$\bar{1}$·6990	1·7	0·2304
18	0·025	$\bar{2}$·3979	0·25	$\bar{1}$·3979	1·25	0·0969	0·004	$\bar{3}$·6020	0·05	$\bar{2}$·6990	0·5	$\bar{1}$·6990	2·0	0·3010
19	0·025	$\bar{2}$·3979	0·25	$\bar{1}$·3979	1·3	0·1139	0·004	$\bar{3}$·6020	0·08	$\bar{2}$·9031	0·5	$\bar{1}$·6990	2·0	0·3010
20	0·03	$\bar{2}$·4771	0·35	$\bar{1}$·5441	1·5	0·1761	0·004	$\bar{3}$·6020	0·1	$\bar{1}$·0000	0·7	$\bar{1}$·8541	2·0	0·3010
21	0·04	$\bar{2}$·6020	0·4	$\bar{1}$·6020	1·5	0·1761	0·004	$\bar{3}$·6020	0·15	$\bar{1}$·1761	0·7	$\bar{1}$·8541	2·0	0·3010
22	0·08	$\bar{2}$·9031	0·6	$\bar{1}$·7782	1·5	0·1761	0·004	$\bar{3}$·6020	0·2	$\bar{1}$·3010	0·7	$\bar{1}$·8541	2·2	0·3424
23	0·08	$\bar{2}$·9031	0·8	$\bar{1}$·9031	2·0	0·3010	0·006	$\bar{3}$·7781	0·2	$\bar{1}$·3010	0·8	$\bar{1}$·9031	2·5	0·3979
24	0·09	$\bar{2}$·9542	0·85	$\bar{1}$·9294	2·2	0·3424	0·006	$\bar{3}$·7781	0·3	$\bar{1}$·4771	0·9	$\bar{1}$·9542	2·5	0·3979
25	0·10	$\bar{1}$·0000	Died	—	2·2	0·3424	0·01	$\bar{2}$·0000	0·4	$\bar{1}$·6020	1·0	0·0000	3·5	0·5441
A.M.	c0·0208		0·207		1·026		0·0028		0·066		0·37		1·38	
G.M.	(median 0·005)		0·071		0·8517		(median 0·0015)		0·015		0·28		1·07	
log G.M.	(log. median $\bar{3}$·699)		$\bar{2}$·8494		$\bar{1}$·9303		(log. median $\bar{3}$·1761)		$\bar{2}$·1815		$\bar{1}$·4494		0·03	
σ	—		0·86		0·2858		—		0·907		0·3557		0·3572	
n	25		23		25		25		25		25		25	

TABLE II.—*Orderly Array of the Individual Responses to Different Doses of F.T. in Guinea-pigs.*

	Exp. 3.								Exp. 4.			
	Group 1 (2 Lf).		Group 2 (10 Lf).		Group 3 (50 Lf)		Group 4 (250 Lf).		Group 1 (2 Lf).		Group 2 (50 Lf).	
Guinea-pig Number.	Titre.	Log. titre.	Titre.	Log. titre.	Titre.	Log. titre.	Titre.	Log. titre.	Titre.	Log. titre.	Titre.	Log. titre.
1	0·0025	$\bar{3}$·3979	0·008	$\bar{3}$·9031	0·008	$\bar{3}$·9031	0·08	$\bar{2}$·9031	0·003	$\bar{3}$·4771	0·025	$\bar{2}$·3979
2	0·004	$\bar{3}$·6020	0·01	$\bar{2}$·0000	0·01	$\bar{2}$·0000	0·08	$\bar{2}$·9031	0·004	$\bar{3}$·6020	0·025	$\bar{2}$·3979
3	0·006	$\bar{3}$·7781	0·01	$\bar{2}$·0000	0·03	$\bar{2}$·4771	0·125	$\bar{1}$·0960	0·004	$\bar{3}$·6020	0·05	$\bar{2}$·6990
4	0·006	$\bar{3}$·7781	0·01	$\bar{2}$·0000	0·03	$\bar{2}$·4771	0·17	$\bar{1}$·2304	0·004	$\bar{3}$·6020	0·05	$\bar{2}$·6990
5	0·006	$\bar{3}$·7781	0·025	$\bar{2}$·3979	0·03	$\bar{2}$·4771	0·25	$\bar{1}$·3979	0·006	$\bar{3}$·7781	0·05	$\bar{2}$·6990
6	0·008	$\bar{3}$·9031	0·03	$\bar{2}$·4771	0·04	$\bar{2}$·6020	0·25	$\bar{1}$·3979	0·006	$\bar{3}$·7781	0·055	$\bar{2}$·7404
7	0·008	$\bar{3}$·9031	0·03	$\bar{2}$·4771	0·05	$\bar{2}$·6990	0·27	$\bar{1}$·4314	0·006	$\bar{3}$·7781	0·07	$\bar{2}$·8451
8	0·008	$\bar{3}$·9031	0·04	$\bar{2}$·6020	0·08	$\bar{2}$·9031	0·27	$\bar{1}$·4314	0·006	$\bar{3}$·7781	0·08	$\bar{2}$·9031
9	0·01	$\bar{2}$·0000	0·04	$\bar{2}$·6020	0·11	$\bar{1}$·0414	0·3	$\bar{1}$·4771	0·008	$\bar{3}$·9031	0·08	$\bar{2}$·9031
10	0·01	$\bar{2}$·0000	0·05	$\bar{2}$·6990	0·11	$\bar{1}$·0414	0·3	$\bar{1}$·4771	0·01	$\bar{2}$·0000	0·125	$\bar{1}$·0969
11	0·01	$\bar{2}$·0000	0·05	$\bar{2}$·6990	0·14	$\bar{1}$·1461	0·3	$\bar{1}$·4771	0·01	$\bar{2}$·0000	0·125	$\bar{1}$·0969
12	0·0125	$\bar{2}$·0969	0·06	$\bar{2}$·7781	0·14	$\bar{1}$·1461	0·35	$\bar{1}$·5441	0·015	$\bar{2}$·1761	0·125	$\bar{1}$·0969
13	0·013	$\bar{2}$·1140	0·06	$\bar{2}$·7781	0·15	$\bar{1}$·1761	0·35	$\bar{1}$·5441	0·025	$\bar{2}$·3979	0·15	$\bar{1}$·1761
14	0·015	$\bar{2}$·1761	0·07	$\bar{2}$·8451	0·15	$\bar{1}$·1761	0·35	$\bar{1}$·5441	0·03	$\bar{2}$·4771	0·17	$\bar{1}$·2304
15	0·020	$\bar{2}$·3010	0·08	$\bar{2}$·9031	0·2	$\bar{1}$·3010	0·35	$\bar{1}$·5441	0·03	$\bar{2}$·4771	0·17	$\bar{1}$·2304
16	0·020	$\bar{2}$·3010	0·09	$\bar{2}$·9542	0·2	$\bar{1}$·3010	0·35	$\bar{1}$·5441	0·035	$\bar{2}$·5441	0·20	$\bar{1}$·3010
17	0·020	$\bar{2}$·3010	0·1	$\bar{1}$·0000	0·2	$\bar{1}$·3010	0·35	$\bar{1}$·5441	0·04	$\bar{2}$·6020	0·20	$\bar{1}$·3010
18	0·025	$\bar{2}$·3979	0·1	$\bar{1}$·0000	0·2	$\bar{1}$·3010	0·4	$\bar{1}$·6020	0·04	$\bar{2}$·6020	0·20	$\bar{1}$·3010
19	0·027	$\bar{2}$·4314	0·1	$\bar{1}$·0000	0·22	$\bar{1}$·3424	0·5	$\bar{1}$·6990	0·04	$\bar{2}$·6020	0·20	$\bar{1}$·3010
20	0·04	$\bar{2}$·6020	0·15	$\bar{1}$·1761	0·25	$\bar{1}$·3979	0·5	$\bar{1}$·6990	0·055	$\bar{2}$·7404	0·30	$\bar{1}$·4771
21	0·05	$\bar{2}$·6990	0·2	$\bar{1}$·3010	0·3	$\bar{1}$·4771	0·5	$\bar{1}$·6990	0·055	$\bar{2}$·7404	0·30	$\bar{1}$·4771
22	0·05	$\bar{2}$·6990	0·35	$\bar{1}$·5441	0·3	$\bar{1}$·4771	0·6	$\bar{1}$·7781	0·07	$\bar{2}$·8451	0·35	$\bar{1}$·5441
23	0·06	$\bar{2}$·7781	0·4	$\bar{1}$·6020	0·35	$\bar{1}$·5441	0·8	$\bar{1}$·9031	0·07	$\bar{2}$·8451	0·45	$\bar{1}$·6532
24	0·10	$\bar{1}$·0000	0·6	$\bar{1}$·7781	0·5	$\bar{1}$·6990	1·25	0·0969	0·08	$\bar{2}$·9031	0·50	$\bar{1}$·6990
25	0·15	$\bar{1}$·1761	Died	—	0·7	$\bar{1}$·8451	1·3	0·1139	0·15	$\bar{1}$·1761	3·0	0·4771
A.M.	0·0274		0·11		0·18		0·414		0·032		0·28	
G.M.	0·0161		0·059		0·112		0·33		0·018		0·14	
log G.M.	$\bar{2}$·2047		$\bar{2}$·7716		$\bar{1}$·05		$\bar{1}$·5232		$\bar{2}$·2553		$\bar{1}$·1461	
σ	0·44		0·505		0·496		0·294		0·50		0·45	
n	25		24		25		25		25		25	

therefore, a breakdown of mathematical linearity in the dose-response line at both extremes of the dose-scale—a not unexpected physiological phenomenon. The lower breakdown dosage for standard Lf 50, 10 mg. $AlPO_4$/ml. P.T.A.P. in our guinea-pigs appears at about 1/250 ml. and for F.T. at 1 Lf or a little less. It is of interest to note that the value of σ doubled when the dose given was nearing the lower breakdown level (Exp. 1 and 2). As regards the upper breakdown dose for P.T.A.P. (Exp. 2), the slope of the line joining the 0·2 mg. (or 1 Lf) and 1·0 mg. (or 5 Lf) points is close to that for the F.T. line.

These findings show that not only is B not necessarily unity, but that the dose-response characteristics of these two forms of diphtheria prophylactic are fundamentally different and the one cannot be expressed in terms of the other.

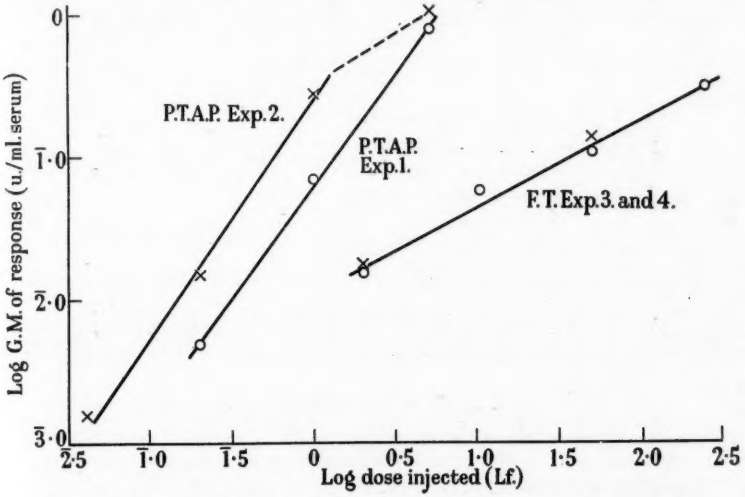


FIG. 1.—Dose-response graphs for P.T.A.P. and F.T. Log G.M./log dose.

If we accept $B = 1\cdot6$ and $0\cdot61$ and $\sigma = 0\cdot35$ and $0\cdot45$ respectively for P.T.A.P. and F.T. we see that b for P.T.A.P. $= 1\cdot6/0\cdot35$ or $4\cdot57$ and b for F.T. $= 0\cdot61/0\cdot45$ or $1\cdot35$. To complete the antigenicity equation given above for P.T.A.P. and F.T. in the guinea-pig, if we let our arbitrary titre At be 0·1 U/ml. (a *geometric mean* titre of At for the calculation of d and the reference *level* for the calculation of b) we obtain :

Antigenicity value	P.T.A.P.	F.T.
B	1·6	0·61
σ	0·35	0·45
b	4·57	1·35
d	about 1 Lf.	33 Lf.

It must be emphasised that, since the value of B is different in the two cases, the ratios of d for these two preparations will vary according to the arbitrary level of response (geometric mean) chosen so that no strict comparison is possible.

It is clear from these findings that P.T.A.P. is superior to its parent F.T. in every particular—B is much greater, σ is slightly smaller and d very considerably smaller. The contrast in the sum total of these antigenic properties is summarised in Fig. 2, where the arbitrary titre has again been chosen at 0·1 U/ml.

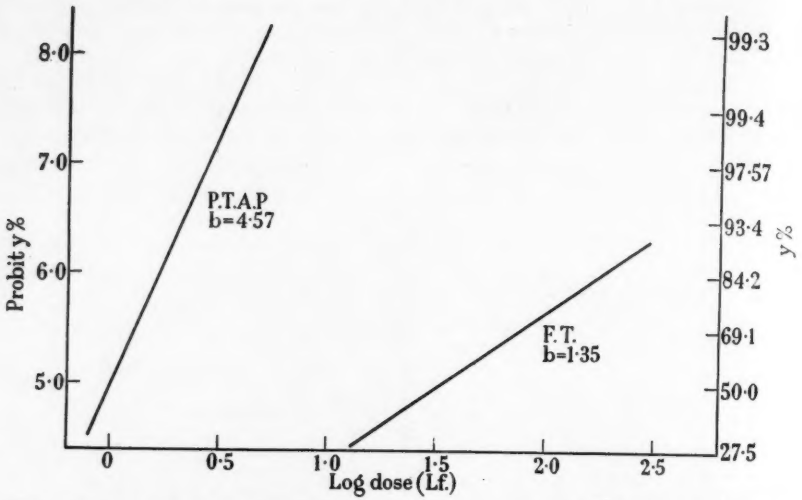


FIG. 2.—Probit y %/log dose regression lines for P.T.A.P. and F.T. Reference titre 0·1 u./ml.

SUMMARY.

The dose-responses of the guinea-pig to P.T.A.P. and its parent purified formol toxoid are not parallel, so that the determination of antigenic potency of one in terms of the other is biometrically invalid.

The range of doses over which an antigenicity equation holds is limited, but of sufficient size to permit accurate determination of three variables.

We are indebted to Dr. P. Armitage, of the Statistical Research Unit of the London School of Hygiene and Tropical Medicine for his helpful advice in the statistical aspects of this paper.

APPENDIX.

Formal proof that

$$B = \sigma\, b \text{ and } \log G.M._{(D)} = \log At_{(d)} + \sigma\, b \log \frac{D}{d}$$

From the cumulative distribution table (Fisher and Yates, 1948, Table I) it follows that, if a normally distributed population has a standard deviation of σ and a mean of M, the percentage of that population which possesses more than an arbitrary measure m may be expressed by the equation

$$M = m + \sigma\,(x \text{ for } 2(100 - y\%) \text{ when } M > m \text{ or}$$

$$M = m - \sigma\,(x \text{ for } 2\,y\%) \text{ when } M < m$$

where

x = the deviation in the normal distribution in terms of standard deviation

$y\%$ = percentage of that population possessing or exceeding the arbitrary measure m.

This relation may also be written as

$$M = m + \sigma \text{ (Probit } y\% - 5) \quad . \quad . \quad . \quad . \quad (a)$$

in which case it is immaterial whether m is greater or less than M.

In the case of antitoxin responses, however, the distribution of titres in a population of similar animals identically treated is lognormal. It is therefore necessary to use geometric means, logs of the individual titres and to calculate the standard deviation from the logs of titres.

Transferring equation (a) to these circumstances we obtain

$$\log G.M. = \log At + \sigma \text{ (Probit } y\% - 5) \quad . \quad . \quad . \quad (b)$$

where

$G.M.$ = geometric mean

At = the arbitrary reference *titre* for probit %.

Equation (b) may be arranged to give

$$\text{Probit } y\% = \frac{\log G.M. - \log At}{\sigma} + 5 \quad . \quad . \quad . \quad . \quad (c)$$

If, however, it requires a dose d to give a *geometric mean* of $At_{(d)}$ and dose D to give another geometric mean $G.M._{(D)}$ we may expand equation (c) as follows:

(*i*) $$\text{Probit } (d) = \frac{\log At_{(d)} - \log At_{(d)}}{\sigma} + 5$$

(*ii*) $$\text{Probit } (D) = \frac{\log G.M._{(D)} - \log At_{(d)}}{\sigma} + 5$$

And since b = the rate of change of probit with respect to dose (log dose in this present case) we obtain, by subtracting (i) from (ii):

$$b = \frac{\dfrac{\log G.M._{(D)} - \log At_{(d)}}{\sigma} + 5 - 5}{\log D - \log d} \quad . \quad . \quad . \quad (d)$$

And from this

$$\sigma b = \frac{\log G.M._{(D)} - \log At_{(d)}}{\log D - \log d} = B \quad . \quad . \quad . \quad (e)$$

where B equals the rate of change of log $G.M.$ in respect of log dose.

Similarly equation (e) may be arranged to give the general equation of antigenicity

$$\log G.M._{(D)} = \log At_{(d)} + \sigma b \log \frac{D}{d} \quad . \quad . \quad . \quad (f)$$

Since $\frac{1}{b} = \sigma_d$ (the s.d. of log *dose*) $B = \frac{\sigma}{\sigma_d}$. Similarly $\sigma = \frac{1}{b_r}$, where b_r = slope

of regression line probit $y\%$ on log *titre*, whence

$$B = \sigma b = \frac{\sigma}{\sigma_d} = \frac{b}{b_r}.$$

REFERENCES.

BARR, MOLLIE AND LLEWELLYN-JONES, MONA—(1951) *Brit. J. exp. Path.*, **32**, 231, Fig. 6.

FISHER, R. A. AND YATES, F.—(1948) 'Statistical Tables for Biological Agricultural and Medical Research.' London (Oliver & Boyd).

HOLT, L. B.—(1950) 'Developments in Diphtheria Prophylaxis.' London (Heinemann), p. 154.—(1953) '3rd int. Biometric Conf.' Bellagio. Abstract of papers, p. 59. —(1955) *Biometrics*, **11**, 83.

PRIGGE, R.—(1953) *Bull. World Hlth Org.*, **9**, 843.—(1954) *Arb. Paul Ehrlich Inst.*, **51**, 108.

RÖMER, P. H. AND SAMES, T.—(1909) *Z. ImmunForsch.*, **3**, 344.

QUANTITATIVE STUDIES IN DIPHTHERIA PROPHYLAXIS. ANTIGENIC INTERACTIONS: I. SIMULTANEOUS INOCULATIONS.

JOAN M. BARNES AND L. B. HOLT.

From the Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, W.2.

Received for publication April 25, 1955.

THE principle that the response of any animal to an antigen is conditioned by its responses to other antigens given previously or at the same time has long been recognised. The interference between the components of injected material was reported by Michaelis (1904) who worked with serum fractions. He showed that the albumen fraction injected alone provoked a large precipitin reaction, while the injection of untreated serum gave rise to precipitin formation only against the globulin. Michaelis called this phenomenon the *Konkurrenz der Antigene*.

During the next few years interest moved to the interference caused by giving one injection soon after another, but by 1926 work had been done on mixed prophylactics. Glenny and Waddington (1926) injected two different mixtures of diphtheria toxoid and scarlet fever toxin into rabbits and reported that a satisfactory response to the diphtheria antigen could be obtained only if the scarlet fever component was small, but they did not determine whether the response to the scarlet fever toxin was adequate at either level of dosage. The effect of adding T.A.B. vaccine to diphtheria toxoid was investigated by Ramon and Zoeller (1926). They reported that their results in man were " at least as good as those obtained with the toxoid alone ". In further experiments in which diphtheria and tetanus toxoids were injected together, they concluded that " the production of tetanus antitoxin is not prejudiced ". The diphtheria titres are not mentioned, nor are any control results with the individual toxoids. In 1927 however Ramon and Zoeller showed more convincingly that the response to tetanus toxoid in man was increased by the simultaneous injection of T.A.B. vaccine and this result was confirmed in a large field trial by Maclean and Holt (1940). Further work by Ramon (1933) on the results of injecting mixed diphtheria/tetanus prophylactics into guinea-pigs has occasioned a great deal of controversy. He compared the antitoxin titres obtained from the injection of diphtheria alone, tetanus alone and the two combined together plus T.A.B. vaccine, and reported that the combined prophylactic resulted in significantly increased titres. It is indeed unfortunate that a fourth group, the two toxoids together without the vaccine, was not included, for in the light of more recent work it seems likely that the adjuvant effect of the vaccine on both toxoids had masked any interference of the toxoids with one another. This interference was clearly shown by Scheibel (1944) working with guinea-pigs. She used diphtheria and tetanus toxoids adsorbed on aluminium hydroxide, varying the doses, but keeping the ratio 2 Lf diphtheria/1 Lf tetanus constant. At all levels the tetanus responses were depressed by the presence of the diphtheria

antigen, significantly so at the lowest level, while the response to the diphtheria antigen was unaffected by the presence of tetanus. The work of Greenberg and Fleming (1947 ; 1948) might be thought to contradict the findings of Scheibel in that the responses they recorded in guinea-pigs to diphtheria toxoid were slightly increased by tetanus toxoid, and very considerably increased by tetanus toxoid with pertussis vaccine or T.A.B. vaccine, and by scarlet fever toxin with pertussis vaccine. Considering only the mixture of diphtheria and tetanus toxoids, Scheibel measured antitoxin titres while Greenberg and Fleming challenged with 10 lethal doses ; this different method of assessment together with the low level of significance obtained in Greenberg and Fleming's results makes a precise comparison difficult. Greenberg and Fleming did not report their tetanus results, so that it is not possible to compare the antigenic balance of their doses in the way that can be done with Scheibel's work. The remainder of the groups immunised by Greenberg and Fleming contained a vaccine and their results have been verified by Farago and Pusztai (1949) and Ungar (1952). Farago and Pusztai used rabbits to show that the addition of *Haemophilus pertussis* vaccine to combined diphtheria and tetanus toxoids gave the same effect as adsorbing the toxoids on aluminium phosphate, and no further advantage was obtained by adding aluminium phosphate in the presence of the vaccine. Ungar (1952) showed that suspensions of several kinds of bacteria including *H. pertussis*, and also collodion particles and yeast cells increased the antigenic efficiency of diphtheria toxoid in rabbits, mice and guinea-pigs to the level obtained with P.T.A.P. Results for the production of pertussis agglutinins when the vaccine is injected alone and with the toxoid were not given. Bousfield and Holt (1954) reported a field trial in which they claimed satisfactory immunity to both diphtheria and pertussis was obtained by using the mixed prophylactic in three spaced doses of 10,000 million organisms of *H. pertussis* vaccine with 30 Lf purified diphtheria toxoid in buffered saline. Schick conversion rates of over 99 per cent were obtained, and in a group of 510 babies having 12 known house contacts with whooping-cough only 4 babies developed the disease during the period of 7–10 months for which they were observed. They verified the adjuvant effect of the vaccine on the toxoid inoculated in children by Schick testing one month after a single dose. The difference between toxoid alone and toxoid with vaccine was not measurable by the Schick method after more than one dose.

Barr and Llewellyn-Jones (1953), working with tetanus and T.A.B. vaccine in guinea-pigs, showed the importance of balancing the antigens in a mixed prophylactic and pointed out that it may be difficult to achieve this balance because of qualitative differences between batches of vaccine. Contrary to other workers, they found, using both the two-dose and large single-dose methods of comparison, that the responses to the tetanus antigen were lower when T.A.B. vaccine was injected with tetanus toxoid than when the toxoid was injected alone. They found an adjuvant effect on one occasion only, when a smaller dose of tetanus toxoid (0·2 Lf instead of 0·5 Lf) was used.

From this evidence it appears that fluid toxoids, such as diphtheria and tetanus toxoid, do compete with one another when injected together, and the response of the animal, presuming that it has not been previously inoculated with either antigen alone, depends on the relative and absolute amounts of each antigen injected, excluding, of course, those cases where there has been previous contact with either antigen. There is also a considerable measure of agreement that

vaccines, like mineral gels, so increase the responses to toxoids that any interference between two toxoids in a mixed prophylactic is masked. Fuller studies on a quantitative basis therefore seemed desirable since the controversy in this case might well be resolved into a quantitative limitation rather than a direct contradiction.

The following experiments were designed in order to investigate some of the quantitative points using diphtheria toxoid and pertussis vaccine in different amounts and ratios. It is realised that the optimum ratio for guinea-pigs should not be presumed to be the optimum for children. Further, since the reported laboratory experiments on the interference between the toxoids were short-term observations, whereas in clinical practice the later results are also important, some long-term experiments with guinea-pigs were done to observe whether the interference effect persisted at a significant level.

EXPERIMENTAL.

The effect of increasing the dose of combined diphtheria-pertussis prophylactic.

The following materials were used: (*a*) purified diphtheria toxoid 70 Lf/ml. and (*b*) purified diphtheria toxoid 35 Lf and 10×10^9 *H. pertussis* organisms per ml. Each was preserved with 0·01 per cent thiomersalate (E.M.T.).

Four groups of 12 guinea-pigs of approximately equal weight (280–300 g.) were injected subcutaneously on the ventral wall. Group I*a* received 1 ml. of (*a*) diluted 1/70, *viz.*, 1 Lf toxoid per dose. Group I*b* received 1 ml. of (*b*) diluted 1/35, *viz.*, 1 Lf toxoid and 285×10^6 organisms per dose. Group II*a* received 1 ml. of (*a*) diluted 1/50, *viz.*, 1·4 Lf toxoid per dose. Group II*b* received 1 ml. of (*b*) diluted 1/25, *viz.*, 1·4 Lf toxoid and 400×10^6 organisms per dose.

Mixture (*b*) is that used in the field trials described by Bousfield and Holt (1954). The recommended vaccine concentration has since been doubled (*British Pharmacopoeia*, 1953) and is the same as has been used for the animals of Group III of the second experiment. The animals were bled 28 days later and re-injected with the same material; 14 days after the second dose they were again bled. In this and subsequent experiments the guinea-pigs were identified by marking them in a colour code, so that the titre of each could be followed. The serum from each individual guinea-pig was titrated to an accuracy of $\pm$ 10 per cent using a micro-technique based on the method of Römer and Sames (1909). Geometric means and standard deviations of the logarithms of titres were calculated for each group. Pertussis agglutinins were not titrated.

The results (Table I) show that the diphtheria antitoxin titres are increased in each case by the addition of pertussis vaccine, the ratios between the means of the groups being greater after two doses than after one dose, and tending to decrease as the dose was increased. Unfortunately only Group I was available for a long-term observation and here the adjuvant effect of the vaccine is still evident 19 weeks after the second injection.

The effect of doubling the dose of H. pertussis *vaccine in a combined diphtheria-pertussis prophylactic.*

From the previous experiment it appeared that the adjuvant effect of *H. pertussis* vaccine on the action of diphtheria toxoid diminished steeply as the dose injected was increased and might indeed disappear at about 3 Lf. Accordingly

TABLE I.—*The Diphtheria Antitoxin Titres of Guinea-pigs Inoculated with Varying Doses of Diphtheria Formol Toxoid and Diphtheria-Pertussis Prophylactics.*

Dose.	Guinea-pig number.	28 days after 1 dose, units/ml.	14 days after 2 doses, units/ml.	19 weeks after 2 doses, units/ml.
I(*a*) 1 Lf diphtheria toxoid	1	0·0025	0·1	0·04
	2	0·005	0·028	0·02
	3	0·08	3·0	1·25
	4	0·005	0·03	0·022
	5	0·0012	0·01	0·006
	6	0·0012	0·08	0·01
	7	0·0012	0·08	0·025
	8	0·005	0·08	0·03
	9	0·0035	0·008	0·004
	10	0·004	0·06	0·07
	11	0·003	0·05	0·04
	12	0·003	0·05	0·05
Geometric mean (*a*)	—	0·0036	0·058	0·03
σ logs	—	0·48	0·63	0·63
I(*b*) 1 Lf diphtheria toxoid + 286 × 10^6 organisms *H. pertussis* vaccine	1	0·5	28·0	4·0
	2	0·015	1·0	0·15
	3	0·15	5·0	0·8
	4	0·05	4·0	2·25
	5	0·017	1·0	0·3
	6	0·006	0·4	0·6
	7	0·18	9·0	4·0
	8	0·025	3·5	1·0
	9	0·055	2·3	0·9
	10	0·015	1·5	0·8
	11	0·03	3·0	1·5
	12	0·08	4·0	2·2
Geometric mean (*b*)	—	0·042	2·85	1·05
σ logs	—	0·55	0·43	0·43
Ratio *b*/*a*	—	11·6	49·2	35·0

Dose.	Guinea-pig number.	28 days after 1 dose, units/ml.	14 days after 2 doses, units/ml.
II(*a*) 1·4 Lf diphtheria toxoid	1	0·01	0·15
	2	0·01	1·25
	3	0·03	1·5
	4	0·006	0·06
	5	0·008	0·05
	6	0·02	1·7
	7	0·035	0·08
	8	0·01	1·0
	9	0·008	0·08
	10	0·01	0·01
	11	0·007	0·45
	12	0·015	1·0
Geometric mean (*a*)	—	0·012	0·24
σ logs	—	0·57	0·73
II(*b*) 1·4 Lf diphtheria toxoid + $\frac{1}{4}$00 × 10^6 organisms *H. pertussis* vaccine	1	0·1	2·0
	2	0·06	2·3
	3	0·2	6·0
	4	0·125	4·5
	5	0·5	11·25
	6	0·13	4·0
	7	0·25	4·5
	8	0·4	11·0
	9	0·3	8·0
	10	0·08	4·5
	11	0·2	11·25
	12	0·5	11·25
Geometric mean (*b*)	—	0·19	5·76
σ logs	—	0·31	0·26
Ratio *b*/*a*	—	15·9	23·8

σ logs = standard deviation of logs of titres.

an experiment was set up in which the dose of toxoid was increased to 3·5 Lf and the proportion of vaccine to toxoid kept constant as in the first experiment. In another group the proportion of vaccine to toxoid was doubled.

The following materials were used : (*a*) purified diphtheria toxoid 70 Lf/ml. + 0·01 per cent E.M.T.; (*b*) purified diphtheria toxoid 35 Lf and 10×10^9 *H. pertussis* organisms per ml. + 0·01 per cent E.M.T.; (*c*) purified diphtheria toxoid 35 Lf and 20×10^9 *H. pertussis* organisms per ml. + 0·01 per cent E.M.T.

TABLE II.—*The Diphtheria Antitoxin Titres of Guinea-pigs Inoculated with Diphtheria-Pertussis Prophylactic containing 3·5 Lf. Diphtheria Toxoid and Varying Amounts of Pertussis Vaccine.*

Dose.	Guinea-pig number.	28 days after 1 dose, units/ml.	14 days after 2 doses, units/ml.
(*a*) 3·5 Lf toxoid. No vaccine	1	0·17	1·25
	2	0·27	7·0
	3	0·025	0·6
	4	0·008	0·0125
	5	0·02	1·0
	6	0·0125	0·6
	7	0·04	2·2
	8	0·617	1·0
	9	0·35	5·0
	10	0·001	0·0025
	11	0·09	2·0
Geometric mean	—	0·033	0·55
σ logs	—	0·64	1·09 (0·75 omitting 10)
(*b*) 3·5 Lf toxoid 1×10^9 organisms *H. pertussis* vaccine	1	0·17	5·0
	2	0·1	7·0
	3	0·1	4·5
	4	0·06	4·0
	5	0·8	22·0
	6	0·55	30·0
	7	0·08	2·0
	8	0·8	20·0
	9	0·1	5·0
	10	0·07	3·0
	11	0·06	7·0
	12	0·08	3·0
Geometric mean	—	0·146	6·45
σ logs	—	0·43	0·37
Ratio *b*/*a*	—	4·4	11·7
(*c*) 3·5 Lf toxoid 2×10^9 organisms *H. pertussis* vaccine	1	0·9	20·0
	2	0·0025	0·7
	3	0·17	5·5
	4	0·01	0·8
	5	0·04	2·5
	6	0·005	0·45
	7	0·02	2·7
	8	0·025	1·25
	9	0·4	7·0
	10	0·006	1·25
	11	0·015	1·0
	12	0·9	22·0
Geometric mean	—	0·039	2·44
σ logs	—	0·91	0·56
Ratio *c*/*a*	—	1·2	4·4

Group I received 1 ml. of (*a*) diluted 1/20, *viz.*, 3·5 Lf toxoid per dose. Group II received 1 ml. of (*b*) diluted 1/10, *viz.*, 3·5 Lf toxoid and 1×10^9 organisms per dose. Group III received 1 ml. of (*c*) diluted 1/10, *viz.*, 3·5 Lf toxoid and 2×10^9 organisms per dose. The injections were given subcutaneously on the ventral wall, using groups of 12 guinea-pigs of approximately equal weight for each mixture.

TABLE III.—*The Diphtheria Antitoxin Titres obtained in Guinea-pigs Inoculated with* (a) *Two Doses of Diphtheria Formol Toxoid;* (b) *One Dose of Combined Diphtheria Toxoid and* H. pertussis *Vaccine followed by a Dose of Diphtheria Formol Toxoid and* (c) *Two Doses of Combined Diphtheria Toxoid and* H. pertussis *Vaccine.*

First dose.	Guinea-pig number.	Responses 28 days after 1st dose, units/ml.	Second dose.	Responses 14 days after 2nd dose, units/ml.	Responses 6 months after 2nd dose, units/ml.
(*a*) 2 Lf toxoid	1	0·01	2 Lf toxoid	0·8	0·45
	2	0·0025		0·008	0·02
	3	0·01		1·25	0·25
	4	0·01		2·0	1·25
	5	0·01		0·5	0·125
	6	0·2		4·5	1·0
	7	0·015		0·3	0·04
	8	0·004		0·4	0·03
	9	0·0125		1·5	0·3
	10	0·15		4·5	2·0
	11	0·01		3·5	1·35
	12	0·0025		0·06	0·008
Geometric mean	—	0·012		0·65	0·19
σ logs	—	0·74		0·81	0·80
(*b*) 2 Lf toxoid 1×10^9 organisms *H. pertussis* vaccine	1	0·2	2 Lf toxoid	7·0	2·0
	2	0·25		8·0	2·2
	3	0·03		1·1	0·3
	4	0·125		2·2	0·6
	5	0·025		2·7	0·55
	6	0·025		4·0	1·0
	7	0·015		4·5	0·8
	8	0·17		30·0	7·0
	9	0·125		7·0	2·0
	10	0·15		Died	—
	11	0·08		3·0	1·35
	12	0·35		7·0	3·5
Geometric mean	—	0·078		4·09	1·3
σ logs	—	0·45		0·36	0·39
(*c*) 2 Lf toxoid 1×10^9 organisms *H. pertussis* vaccine	1	0·3	2 Lf toxoid 1×10^9 organisms *H. pertussis* vaccine	9·0	2·7
	2	0·1		6·0	3·0
	3	0·09		3·0	0·3
	4	0·125		4·5	1·0
	5	0·06		3·5	2·0
	6	0·22		8·0	5·5
	7	0·006		2·7	3·0
	8	0·5		15·0	1·5
	9	0·125		5·0	1·1
	10	0·08		2·7	1·5
	11	0·25		17·0	3·0
	12	0·015		2·0	0·45
Geometric mean	—	0·094		5·19	1·6
σ logs	—	0·53		0·3	0·36

The blood samples were taken and sera titrated individually as in the previous experiment, and the results are shown in Table II. The results from this second experiment should be examined from two points of view.

First, Groups I and II represent a continuation of the first experiment and show, as expected, that the adjuvant effect of the vaccine on diphtheria antitoxin production is much less marked at the higher dose level, for the ratios of antitoxin geometric means are now reduced to 4·4 in respect of primary responses and to about 12 for secondary responses. It may be noted that the responses from the 1·4 Lf plus vaccine mixture is not significantly different from the 3·5 Lf plus vaccine mixture in respect of both primary and secondary responses.

Secondly, Group III compared with Groups I and II shows important differences in that by doubling the ratio of vaccine to toxoid at the 3·5 Lf level the antitoxin response to primary stimulation is only equal to the " toxoid alone " control and is much *less* than the response in animals which received the same amount of toxoid but half the vaccine dosage. The adjuvant effect of the vaccine at this toxoid level with double strength vaccine is seen only in the secondary responses (Groups I and III) where the ratio is reduced to 4·4.

Further work is proceeding to define the limit of this adjuvant effect. Pertussis agglutinins were not titrated.

To find the effect of adding H. pertussis *vaccine to the primary stimulus only, compared with its addition to both primary and secondary doses of diphtheria toxoid.*

Since the ratios of the means of diphtheria antitoxin titres between the group receiving vaccine with toxoid and those receiving toxoid alone were at each dose level greater after the second dose than after the first, it was decided to investigate the effect of adding the vaccine to the primary dose only.

The following materials were used : (*a*) purified diphtheria toxoid diluted to 2 Lf/ml.; (*b*) *H. pertussis* vaccine containing 1×10^9 organisms/ml. + 0·01 per cent E.M.T. together with toxoid (*a*). All doses were diluted to 1 ml. in saline and injected subcutaneously on the ventral surface of three groups of twelve guinea-pigs. Blood samples were taken and sera titrated individually as in the previous experiments. The results are given in Table III.

They show that the adjuvant effect of the pertussis vaccine on diphtheria toxoid is exerted in the primary dose and that no significant difference in diphtheria antitoxin titres is obtained either at the secondary peak or at the base line by adding vaccine to the secondary dose. A fourth group in which vaccine was added to the secondary dose but not to the primary should have been included in this experiment. Isolated observations suggest that this results in titres not significantly different from those obtained when both doses consist of toxoid alone.

To investigate the interference between diphtheria and tetanus toxoids in the presence of H. pertussis *vaccine.*

Since *H. pertussis* vaccine enhances the antigenicity of small doses of purified diphtheria toxoid, while that of tetanus toxoid is enhanced by the addition of T.A.B. vaccine (Maclean and Holt, 1940), it was decided to investigate the interaction of diphtheria and tetanus toxoids with pertussis vaccine. Further, in order to assess the practical importance of the interaction, the experiment was continued

for six months after the second injection in order that the base lines could be compared.

The following materials were used: (*a*) purified diphtheria toxoid diluted to 1·5 Lf/ml.; (*b*) crude tetanus toxoid diluted to 1 Lf/ml.; (*c*) *H. pertussis* vaccine containing 500×10^6 organisms/ml. Group I received diphtheria-pertussis,

TABLE IV.—*The Diphtheria and Tetanus Antitoxin Titres of Guinea-pigs Inoculated with Diphtheria-Pertussis, Tetanus-Pertussis and Diphtheria-Tetanus-Pertussis Prophylactics.*

Dose.	Guinea-pig no.	Diphtheria antitoxin titres, units/ml. 28 days after 1st dose.	14 days after 2nd dose.	6 months after 2nd dose.	Tetanus antitoxin titres, units/ml. 14 days after 2nd dose.	6 months after 2nd dose.
(*a*) Diphtheria 1·5 Lf 500×10^6 organisms *H. pertussis* vaccine	1	0·06	2·0	0·25	—	—
	2	0·004	1·0	0·1	—	—
	3	0·06	6·0	2·0	—	—
	4	0·08	4·5	1·25	—	—
	5	0·004	1·0	0·125	—	—
	6	0·025	3·0	2·5	—	—
	7	0·2	12·5	2·5	—	—
	8	0·15	4·5	2·5	—	—
	9	0·25	6·0	0·8	—	—
	10	0·025	5·0	0·8	—	—
	11	0·02	1·5	0·6	—	—
	12	0·06	2·5	0·4	—	—
Geometric mean	—	0·04	3·17	0·72	—	—
σ logs	—	0·59	0·34	0·50	—	—
(*b*) Tetanus 1 Lf 500×10^6 organisms *H. pertussis* vaccine	1	—	—	—	0·5	0·05
	2	—	—	—	0·02	<0·005
	3	—	—	—	0·1	0·03
	4	—	—	—	0·02	<0·005
	5	—	—	—	0·02	0·005
	6	—	—	—	0·5	0·005
	7	—	—	—	0·02	0·005
	8	—	—	—	0·03	0·02
	9	—	—	—	0·5	0·3
	10	—	—	—	0·5	0·5
	11	—	—	—	0·4	0·005
	12	—	—	—	0·01	<0·005
Geometric mean	—	—	—	—	0·08	Median 0·005
σ logs	—	—	—	—	0·70	
(*c*) Diphtheria 1·5 Lf tetanus 1 Lf 500×10^6 organisms *H. pertussis* vaccine	1	0·04	2·0	0·4	0·3	0·05
	2	0·004	0·2	0·025	0·01	0·005
	3	0·02	0·8	0·15	0·01	<0·005
	4	0·02	0·3	0·08	0·05	0·005
	5	0·125	6·0	1·0	0·02	0·02
	6	0·08	1·5	0·5	0·05	0·01
	7	0·02	0·25	0·025	1·0	0·01
	8	0·125	10·0	0·8	0·1	<0·005
	9	0·03	2·0	0·4	0·05	0·01
	10	0·02	0·8	0·3	0·2	0·5
	11	0·08	2·0	4·0	1·0	0·5
	12	0·025	0·2	0·015	0·05	0·005
Geometric mean	—	0·03	0·85	0·21	0·08	Median 0·01
σ logs	—	0·43	0·57	0·74	0·68	

Group II tetanus-pertussis and Group III received diphtheria-tetanus-pertussis prophylactics. The materials were available in concentrated form and were diluted in saline to give the above doses in 1 ml. The three groups of twelve guinea-pigs were injected on the ventral surface and their sera titrated individually. Since the sera of Group III were to be titrated for both diphtheria and tetanus antitoxins, the diphtheria titrations were taken to an accuracy of $\pm$ 20 per cent and the tetanus titrations, using a microtechnique based on the method of Glenny and Stevens (1938), to an accuracy of $\pm$ 50 per cent. The results are shown in Table IV.

Unfortunately, this batch of crude tetanus toxoid had deteriorated and become an extremely inefficient antigen. In spite of this the diphtheria responses of the group receiving the combined diphtheria tetanus antigen were significantly lower after the second dose than those normally obtained with diphtheria toxoid alone both at the peak and the base line, while the responses to the first dose were not affected.

DISCUSSION.

The adjuvant action of *H. pertussis* vaccine on the antigenic action of diphtheria toxoid is most marked with small doses and diminishes rapidly as the dose is increased. It may be postulated that the action of the vaccine is to decrease the size of the dose needed to stimulate the antibody-producing cell receiving both components (Holt, 1950). It is difficult to equate the function of the vaccine with that of a mineral gel carrier, such as aluminium phosphate, since the toxoid is not adsorbed to the vaccine, while it must be adsorbed to the gel for it to increase the antigenic efficiency of the toxoid. Miss W. A. Boyce showed in unpublished work in this laboratory that the antitoxin titres obtained by injecting toxoid with, but not adsorbed on, aluminium phosphate are comparable with those obtained by injecting toxoid alone. Both in the case of toxoid with pertussis vaccine and toxoid adsorbed on aluminium phosphate the important finding seems to be the greatly increased efficiency of the primary stimulus.

The injection of two fluid toxoids together results in some competition, giving a response reflecting not only the relative doses of the two antigens but also, possibly, the purity of those antigens. For instance the response to an undamaged highly purified toxoid might be expected to be considerably reduced by the presence of another antigen, while the response to a crude toxoid in which the required antigen is already competing with many antigenic impurities may be little affected by the presence of one more antigen.

The controversy about adjuvance and interference in combined prophylactics may perhaps be resolved into a consideration of quantities. There is evidence of interference between fluid toxoids injected simultaneously even at those levels of dosage needed to establish satisfactory immunity. Vaccines have at these levels only an adjuvant effect on the antigenicity of the toxoids, possibly by considerably reducing the size of the minimal stimulating dose of toxoid. When the toxoid dose is already adequate, or more than adequate, then the adjuvant effect of the vaccine is not demonstrated and there is some evidence that at higher dosage levels of vaccine there may even be interference with antitoxin production.

SUMMARY.

In the guinea-pig the adjuvant effect of the vaccine on the antigenicity of the toxoid component of a liquid diphtheria toxoid-*H. pertussis* mixture is most

marked when the dose inoculated is very small. This adjuvant effect rapidly decreases as the dose is increased. The adjuvant effect of the vaccine on the antitoxin responses is more marked at the secondary than at the primary response stage, but this appears to be due to the effect of the vaccine in the *primary* stimulus.

There is evidence that the adjuvant effect of vaccine may be greatly reduced simply by doubling the ratio of vaccine to toxoid. This is of possible clinical importance since it is found in the guinea-pig that the recently revised ratio of vaccine to toxoid used in combined diphtheria-pertussis prophylactics has a greatly reduced adjuvant effect compared with the former mixture which contained only half as many bacteria.

The response to the diphtheria antigen in a triple diphtheria-tetanus-pertussis prophylactic is greater than that obtained with diphtheria toxoid alone but less than with the diphtheria-pertussis mixture. The difference was still significant six months after the second injection even though the tetanus toxoid was itself an inefficient antigen.

REFERENCES.

Barr, Mollie and Llewellyn-Jones, Mona—(1953) *Brit. J. exp. Path.*, **34**, 12.

Bousfield, G. and Holt, L. B.—(1954) *Med. Offr.*, **92**, 289.

'British Pharmacopoeia'—(1953) p. 604.

Farago, F. and Pusztai, S.—(1949) *Brit. J. exp. Path.*, **30**, 572.

Glenny, A. T. and Waddington, Hilda—(1926) *J. Path. Bact.*, **29**, 118.

Idem and Stevens, Muriel—(1938) *J.R. Army med. Cps*, **70**, 308.

Greenberg, L. and Fleming, D. S.—(1947) *Canad. publ. Hlth J.*, **38**, 279.—(1948) *Ibid.*, **39**, 131.

Holt, L. B.—(1950) 'Developments in Diphtheria Prophylaxis.' London (Heinemann), p. 144.

Maclean, I. H. and Holt, L. B.—(1940) *Lancet*, ii, 581.

Michaelis, L.—(1904) *Dtsch med. Wschr.*, **30**, 1240.

Ramon, G.—(1933) *C.R. Acad. Sci., Paris*, **197**, 1361.

Idem and Zoeller, C.—(1926) *C.R. Soc. Biol., Paris*, **94**, 106.—(1927) *Ann. Inst. Pasteur*, **41**, 803.

Römer, P. H. and Sames, T.—(1909) *Z. ImmunForsch.*, **3**, 344.

Scheibel, Inga F.—(1944) *Acta path. microbiol. scand.*, **21**, 130.

Ungar, J.—(1952) *Proc. R. Soc. Med.*, **45**, 674.

HAEMIN-ISONIAZID INTERACTION AND THE EFFECT OF HAEMIN IN "REVIVING" ISONIAZID-TREATED TUBERCLE BACILLI.

R. KNOX AND R. C. WOODROFFE.

From the Department of Bacteriology, Guy's Hospital Medical School, London, S.E.1.

Received for publication April 20, 1955.

FISHER (1954*a*, *b*) stated that haemin, which acted as an apparent growth factor for certain isoniazid-resistant strains of tubercle bacilli, also powerfully antagonised the action of the drug on isoniazid-sensitive strains. Knox (1955) confirmed and extended Fisher's observation. Both these workers, though aware of Gray's observation (Gray, 1953) that isoniazid reacted with haemin in alkaline conditions, were at first unable to detect significant destruction of isoniazid by haemin in uninoculated media at pH 6·8. However, Youmans (personal communication) and Cohn, Oda, Kovitz and Middlebrook (1954) indicated that significant destruction of isoniazid in the presence of haemin could occur in uninoculated media. Knox, Albert and Rees (1955) in a preliminary communication stated that while haemin and isoniazid reacted to form a purple haemochromogen stable in the absence of air, in the presence of air haemin catalysed the oxidative destruction of isoniazid to di-*iso*nicotinoyl hydrazine and *iso*nicotinic acid, thus finding the same products as were reported independently by Krüger-Thiemer (1955), though suggesting a different mechanism for their formation. Part of this work is more fully described in a separate communication by Albert and Rees (1955) and part of it in the present paper. This describes the results of microbiological, spectroscopic, spectrophotometric and chromatographic studies in this laboratory of the haemin-isoniazid interaction and also some observations on the reversibility of isoniazid inhibition by haemin.

METHODS.

Materials.

Haemin (Light's Chemicals Ltd., Colnbrook, Bucks) was dissolved in a few drops of N-NaOH and made up to the required concentration in Fisher's medium or in about 0·025 M phosphate buffer pH 6·8 (Fisher, 1952), but in some experiments 0·05 M, pH 7·5 (Albert and Rees, 1955). Isoniazid (Smith and Nephew Research, Ltd.) was added to the required concentration. Samples of haemin-isoniazid mixtures were treated immediately and at intervals with HCl (final concentration 0·05 N) and centrifuged. The supernatant fluid was assayed microbiologically, by paper chromatography or by spectrophotometry. The precipitated haemin was re-dissolved in a few drops of N-NaOH and made up to its original concentration in buffer. It was investigated microbiologically, by spectroscopy or by spectrophotometry.

Microbiological assay.

This was used for estimating isoniazid in concentrations which could not be detected by other methods. With an isoniazid-sensitive strain of *Mycobacterium tuberculosis* H37Rv as little as 10^{-7} M (0·0137 μg./ml.) isoniazid could be detected. Haemin could be assayed to nearly 10^{-6} M (0·65 μg./ml.) by using an isoniazid-resistant haemin-"dependent" variant of this strain (Knox, 1955).

Spectroscopy.

A direct vision spectroscope (R. & J. Beck, Ltd.) was used; it had an illuminated scale graduated in mμ and a comparison prism for quantitative work when necessary.

Chromatography.

Both ascending and descending methods were used, with Whatman's No. 1 paper and with either *n*-butanol—HCl (20 ml. of concentrated HCl added to 100 ml. *n*-butanol and saturated with water) or 0·5 M-NH_4Cl in water as solvent. Quantities of 0·05 ml. were applied to the paper which was run 16 hr. for butanol and 6 hr. for NH_4Cl and then after drying examined in ultraviolet light. Two lamps were used, one emitting principally at 254 mμ. (Thermal Syndicate mercury burner T/M4/369) with a Chance Bros. OX7 filter, the other emitting principally at 365 mμ. through a Wood's glass (G.E.C. model B.T.H./MRG 50). Ultraviolet absorbing substances showed up against the fluorescent paper as dark spots. They were identified and roughly estimated by comparison with "markers" of known standard solutions which were always included as controls.

Spectrophotometry.

For the visible range a Unicam SP. 350 absorptiometer was used, for the ultraviolet range a Unicam SP. 500 with hydrogen lamp power supply unit (Unicam Instruments, Cambridge, Ltd.).

RESULTS.

Disappearance of Isoniazid in Presence of Haemin.

Microbiological assay.

The disappearance of isoniazid from haemin-isoniazid mixtures in Fisher's buffer or medium (Fisher, 1952) was followed by removing samples and, after treating them as described above, assaying them for isoniazid microbiologically. In shaken flasks or open tubes isoniazid disappeared more rapidly than in evacuated Thunberg tubes (Table I). The rate at which it disappeared varied in different experiments. On several occasions the disappearance seemed to be more rapid in an open test-tube than in a shaken flask, but this was not found consistently. The reaction occurred at 37° and at room temperature.

TABLE I.—*Effect of Aeration on Disappearance of Isoniazid in Presence of Haemin* ($0·2 \times 10^{-3}$ M).

Isoniazid remaining (percentage of initial concentration).

Initial isoniazid concentration $0·2 \times 10^{-3}$ M.

Room temperature.	Thunberg tube.	Open tube.	Flask.
3 hr.	100	50	50
6 „	100	—	12·5
24 „	100	3	0·75
48 „	50	0·4	0·2

Initial isoniazid concentration 10^{-3} M.

Room temperature.	Thunberg tube.	Open tube.	Flask.
3 hr.	100	100	100
6 „	100	50	100
24 „	100	50	25
48 „	100	12·5	0·75

In one experiment at 37° isoniazid in concentrations ranging from $0{\cdot}5 \times 10^{-1}$ M to 10^{-4} M was exposed to the action of haemin ($0{\cdot}5 \times 10^{-3}$ M) at 37° for 24 hr. It was found that the amount of isoniazid which disappeared was between 10^{-2} M and 10^{-3} M. At the end of this time the haemin, after contact with each concentration of isoniazid, was separated, re-dissolved and assayed microbiologically. Whatever the concentration of isoniazid to which it had been exposed, the reconstituted haemin was in each case as active as a control sample of untreated haemin in supporting the growth of the isoniazid-resistant strain of *Myco. tuberculosis* H37Rv. Haemin ($0{\cdot}8 \times 10^{-4}$ M) was allowed to act upon isoniazid ($0{\cdot}7 \times 10^{-4}$ M) in phosphate buffer at pH 6·8 and after 24 hr. fresh isoniazid in the same final concentration was added to the precipitated and reconstituted haemin and the process was performed three times. Microbiological assay showed that in each case the isoniazid had been reduced to less than 10^{-7} M (being undetectable by microbiological assay), while the haemin was still as efficient as initially in promoting the growth of the resistant variant of H37Rv which required it. These experiments strongly suggested that the action of haemin was catalytic.

Di-*iso*nicotinoyl hydrazine and *iso*nicotinic acid, which are among the products formed when isoniazid is oxidised by haemin (Knox *et al.*, 1955), were assayed for microbiological activity against the isoniazid-sensitive and isoniazid-resistant strains of H37Rv. Table II shows that di-*iso*nicotinoyl hydrazine was 500–1000 times less active than isoniazid against the isoniazid-sensitive strain, the minimum inhibitory concentration (MIC) being about $2\text{–}4 \times 10^{-4}$ M (5–10 μg./ml.). Its formation from isoniazid could therefore interfere with microbiological assays, but probably only if concentrations of that order were reached. *Iso*nicotinic acid showed no inhibitory action against either strain at 200 μg./ml. (about 2×10^{-3} M).

TABLE II.—*Effect of Isoniazid, Di-*isonicotinoyl Hydrazine *and* Isonicotinic *Acid on Growth of* Myco. tuberculosis *H37Rv.*

	Minimum inhibitory concentration.			
	Isoniazid-sensitive strain.		Isoniazid-resistant strain.	
	μg./ml.	Molarity (approx.).	μg./ml.	Molarity (approx.).
Isoniazid	0·01	10^{-7}	10	10^{-4}
Di-*iso*nicotinoyl hydrazine .	5–10	$2\text{–}4 \times 10^{-4}$	>200	10^{-3}
*Iso*nicotinic acid . .	>200	2×10^{-3}	>200	2×10^{-3}

Paper chromatography.

With butanol-HCl as solvent, *iso*nicotinic acid (R_F 0·3) was readily separated from isoniazid (R_F 0·05) and *iso*nicotinamide (R_F 0·12). These showed up as dark spots in the ultraviolet, both at 254 mμ. and at 365 mμ. Isoniazid in addition showed as a yellow spot visible in daylight and in ultraviolet. Isoniazid-containing solutions which had been treated with HCl before chromatography sometimes showed, in the position of *iso*nicotinamide (R_F 0·12), an ultraviolet absorbent spot, which differed from that of *iso*nicotinamide itself by its yellow colour in visible light. Di-*iso*nicotinoyl hydrazine showed an ultraviolet absorbent spot in about the position of isoniazid but without its characteristic yellow colour.

*Iso*nicotinic acid was readily identified as one of the products formed when samples of haemin-isoniazid mixtures were examined by the method described.

Di-*iso*nicotinoyl hydrazine however was not detected as one of the products until 0·5 M-NH_4Cl was used as solvent, as recommended by Albert and Rees (1955). With this solvent the R_F was, for isoniazid 0·7, for di-*iso*nicotinoyl hydrazine 0·9, for *iso*nicotinic acid 0·8, but *iso*nicotinamide had the same R_F as di-*iso*nicotinoyl hydrazine. It could be distinguished from *iso*nicotinamide because it gave a yellow fluorescence at 365 mμ but not 254 mμ. With this solvent, *iso*nicotinic acid showed absorption at 254 mμ but not at 365 mμ.

In several experiments the formation of *iso*nicotinic acid from isoniazid was followed by taking daily samples from aerated haemin-isoniazid mixtures and assaying the supernatant fluids (as described above) for isoniazid and *iso*nicotinic acid. In one experiment with 10^{-2} M haemin and 10^{-1} M isoniazid, *iso*nicotinic acid continued to increase for about 2 days, reaching then a concentration equivalent to about half the isoniazid originally present. After that the *iso*nicotinic acid concentration remained steady and the isoniazid slowly disappeared. Addition of fresh isoniazid however gave a further increase in the production of *iso*nicotinic acid.

Isoniazid Haemochromogen.

Spectroscopy and spectrophotometry.

With a direct vision spectroscope it was found that isoniazid readily formed a haemochromogen with haemin. On adding solid isoniazid to a solution of 10^{-3} M haemin in 5×10^{-3} N-NaOH a purple colour immediately developed quite distinct from the orange-red colour of pyridine haemochromogen. Isoniazid haemochromogen showed strong absorption bands at about 560 and 525 mμ. It was formed most readily at high pH and with large excess of isoniazid. But the characteristic change of colour and absorption bands appeared also with lower molarities of isoniazid and at lower pH (for example, in Fisher's buffer at pH 6·8), provided a reducing agent such as $Na_2S_2O_4$ was added. When haemin and isoniazid were present in these conditions in equimolar amounts (for example, 10^{-3} M) in addition to the haemochromogen bands the main band of reduced haemin at about 580 mμ could also be seen. In such a system, as the proportion of isoniazid to haemin increased, the reduced haemin band disappeared and the purple colour and the haemochromogen bands intensified. This suggested that a large excess of isoniazid was necessary in order to convert any given concentration of haemin completely into haemochromogen. This suggestion was confirmed by the absorption spectra in the visible range given by mixtures of haemin ($0{\cdot}16 \times 10^{-3}$ M) with isoniazid in concentrations ranging from $0{\cdot}5 \times 10^{-6}$ M to $0{\cdot}5 \times 10^{-1}$ M. Apart from the strong absorption in the region of 400 mμ (Soret band), the peak absorption was at about 560 mμ, and the highest peak of all was given by the highest concentration of isoniazid tested ($0{\cdot}5 \times 10^{-1}$ M).

Isoniazid-haemochromogen formation occurred when isoniazid and reduced haemin were mixed in Thunberg tubes which had been evacuated and filled with oxygen-free nitrogen—conditions in which oxidative destruction of isoniazid by haemin was negligible. The haemochromogen was stable in absence of air, but the haemochromogen bands disappeared on admission of air and the colour changed from purple to brown. It has not yet been possible to establish whether the haemochromogen dissociates into free haemin and isoniazid, or the products of its oxidation, or simply into an oxidised form (haemichrome). The characteristic haemochromogen formed again immediately on addition of $Na_2S_2O_4$.

Di-*iso*nicotinoyl hydrazine formed a purple haemochromogen with haemin, but the haemochromogen bands were weaker and formed much more slowly than with isoniazid. No haemochromogen bands were seen with *iso*nicotinic acid-haemin mixtures.

Reversibility of isoniazid inhibition of Myco. tuberculosis *by haemin.*

Haemin thus undoubtedly reacts with and in certain conditions catalyses the oxidation of isoniazid in uninoculated media. At first sight this would seem to dispose of the view that haemin competitively antagonises the action of isoniazid on tubercle bacilli. Experiments on the reversibility of isoniazid inhibition by haemin have however given some interesting results.

Sets of tubes of Fisher's medium containing a range of isoniazid concentrations from about 10^{-4} to less than 10^{-7} M were inoculated with *Myco. tuberculosis* H37Rv, and haemin (about 10^{-4} M) was added at intervals (Table III). It can be

TABLE III.—*Reversibility of Isoniazid Inhibition of* Myco. tuberculosis *by Haemin.*

Medium.	Time of adding haemin.	Minimum inhibitory concentration of isoniazid (μg./ml.) after incubation for: 1 week from start of experiment.	4 weeks after adding haemin.
Fisher	None added	0·01	0·05
Fisher + haemin	At start	20	20
Ditto	8 hr.	20	20
,,	15 ,,	20	20
,,	1 day	1	20
,,	2 days	0·01	20
,,	3 ,,	0·01	0·05
,,	5 ,,	0·01	0·05
,,	7 ,,	0·01	0·05
,,	14 ,,	0·01	0·1
,,	21 ,,	0·01	0·05 (3 wk.)
,,	28 ,,	0·01	0·05 (2 wk.)

seen that a control set of tubes which received no haemin showed normal sensitivity to about 10^{-7} M isoniazid (0·0137 μg./ml.) after 1 week's incubation and to a slightly higher level after 4 weeks' incubation. The set of tubes which received haemin immediately showed normal reversal of isoniazid inhibition. But if addition of haemin was delayed, rapid reversal of isoniazid inhibition no longer occurred, though delayed reversal did occur. If the addition was delayed for 3 days or more, growth did not occur at all in isoniazid concentrations much above 10^{-7} M. In other words, the inhibitory action of isoniazid on *Myco. tuberculosis* was fully reversible by haemin only if the haemin was added within a few hours of inoculation into isoniazid-containing medium. These results do not agree with those reported by Fisher (1954*c*) who found that, even after prolonged exposure to high concentrations of haemin, tubercle bacilli could be "revived" by haemin. In some of Fisher's experiments "resting" cells were used, and his conditions were therefore different from those in our experiments in which the cells were freshly inoculated into nutrient medium containing different concentrations of

isoniazid. But in other experiments the conditions used by Fisher seem to have been the same as ours, yet the results are different.

This discrepancy is hard to explain, but it seems that the nature of the medium into which the cells are inoculated may be important. In one experiment tubercle bacilli were inoculated into two types of medium—Fisher's (complete) medium and the same medium (incomplete) from which the asparagine and glycerol were omitted. The cells which had been exposed to high concentrations of isoniazid in the complete medium showed the usual failure to " revive " when haemin was added more than 24 hr. after inoculation, whereas cells which had been exposed to isoniazid in the incomplete medium could be revived by the addition of haemin (together with asparagine and glycerol) as long as 7 days after inoculation. In other words, it seemed that tubercle bacilli survived exposure to isoniazid longer in a non-nutrient than in a nutrient medium. Since these experiments were performed in liquid media, they give no information about the relative survival rates of different organisms in the bacterial population during the period of exposure to isoniazid. It is planned to repeat and extend these experiments to include viable counts, so as to determine the number of cells surviving exposure to isoniazid in complete and incomplete media.

DISCUSSION.

It is now clear that the apparent " antagonism " between haemin and isoniazid described by Fisher can be partly explained by chemical interaction between them ; here there are two groups of observations which at first seemed to be in conflict. The formation of a co-ordination compound (haemochromogen) suggested a stoichiometric reaction in which, by analogy with other haemochromogens, probably two molecules of isoniazid would combine with one of haemin. The microbiological and chromatographic evidence indicated a catalytic oxidase- or peroxidase-like action of haemin. Many haemochromogens, however, are much more efficient catalysts of oxidation processes than haemin itself (Barron, 1939). It seems likely that isoniazid combines with haemin to form a haemochromogen which, in favourable conditions, catalyses the continued oxidation of the remaining isoniazid by oxygen. It is possible that the oxidative destruction proceeds most efficiently when both oxygen and a reducing system are present, so that the haemochromogen alternates between the oxidised and the reduced form, somewhat in the same way as a reducing system is necessary for the cytochrome-cytochrome oxidase system.

The experiments on reversal, however, show that haemin and isoniazid can still be regarded in a sense as antagonists for an unknown site in the tubercle bacillus. We do not know whether haemin penetrates into it, but it seems clear that isoniazid can reach a site in the tubercle bacillus at which it is inaccessible to oxidative destruction by haemin, or else can produce damage to the metabolism of the tubercle bacillus no longer reversible by haemin.

It is possible that when tubercle bacilli are exposed to isoniazid in a complete medium, many of their metabolic processes are unimpaired, though actual cell multiplication is suppressed. If isoniazid interfered with the formation of porphyrin enzymes of mycobacteria, then hydrogen peroxide might accumulate in toxic amounts, and haemin could " revive " the cells either directly by destroying peroxide or indirectly by destroying isoniazid which blocks the formation of peroxide-destroying enzymes. It is therefore still possible that, as was suggested

earlier (Knox, 1955), the site of action of isoniazid on drug-sensitive organisms is the same " locus " which is deficient in certain drug-resistant organisms, and is closely connected with removal of peroxide by peroxidase or catalase.

SUMMARY.

The effect of haemin in antagonising the action of isoniazid can be partly explained by chemical interaction between the two substances known to occur in uninoculated media. Two lines of investigation were followed—one which showed the rôle of haemin in catalysing the oxidation of isoniazid by molecular oxygen and the other which established the formation of a co-ordination compound between haemin and isoniazid (haemochromogen). These results suggested that the true catalyst of isoniazid oxidation might be the haemochromogen rather than haemin.

The effect of haemin in " reviving " isoniazid-treated tubercle bacilli can also be partly explained by haemin-isoniazid interaction. However cells exposed to isoniazid in a nutrient medium could not be " revived " by haemin if the addition of the haemin was delayed for 24 hours or sometimes less. This suggested that isoniazid rapidly reached a site in the tubercle bacillus at which it caused damage no longer reversible by concentrations of haemin adequate to destroy extracellular isoniazid.

We are grateful to Dr. E. F. Hartree for his valuable help and suggestions in the spectroscopic work, and to Dr. G. P. Youmans, Dr. M. W. Fisher, Professor A. Albert, Professor C. Gray and Professor C. Rimington for many valuable discussions. We are also indebted to Mr. D. E. Seymour of Smith & Nephew Research, Ltd. for supplies of isoniazid and other nicotinic acid derivatives, and to Mrs. D. Needs for technical assistance.

REFERENCES.

ALBERT, A. AND REES, C. W.—(1955) *Biochem. J.*, in press.
BARRON, E. S. G.—(1939) *Cold Spr. Harb. Symp. quant. Biol.*, **7**, 154.
COHN, M. L., ODA, U., KOVITZ, C. AND MIDDLEBROOK, G.—(1954) *Amer. Rev. Tuberc.*, **70**, 465.
FISHER, M. W.—(1952) *Ibid.*, **66**, 626.—(1954*a*) *Ibid.*, **69**, 469.—(1954*b*) *Ibid.*, **69**, 797.—(1954*c*) *Proc. Soc. exp. Biol., N.Y.*, **85**, 538.
GRAY, C. T.—(1953) *Proc. VIth int. Congr. Microbiol., Rome*, in press.
KNOX, R.—(1955) *J. gen. Microbiol.*, **12**, 191.
Idem, ALBERT, A. AND REES, C. W.—(1955) *Nature, Lond.*, **175**, 1084.
KRÜGER-THIEMER, E.—(1955) *Naturwissenschaften*, **42**, 47.

THE EFFECT OF X-RAYS ON THE OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA IN RELATION TO NUCLEAR DAMAGE.

D. W. VAN BEKKUM AND O. VOS.*

From the Medical Biological Laboratory of the National Defence Research Council T.N.O., Rijswijk, The Netherlands.

Received for publication April 26, 1955.

AMONG the cellular processes which have been found to be impaired after irradiation, those located in the nuclei are considered to be the most radiosensitive and many of the degenerative features which are encountered in irradiated cells have been attributed to this nuclear damage. The nuclear functions of DNA synthesis and mitosis have been widely studied after irradiation and in some tissues were found to be inhibited as early as 30 minutes after the end of the irradiation. Relatively little attention has been paid to cytoplasmic changes after irradiation, although the literature contains reports on morphological signs of radiation damage to the cytoplasm. Several authors have described structural changes in the mitochondria, in some cases within the first hour after irradiation (Hirsch, 1931; Ludford, 1932; Nadson and Rochlin, 1934). Although these changes, which consist mainly of a transformation of the filamentous structure into the granular form, do not seem to be specific, the same holds for most of the nuclear alterations after irradiation. According to present biochemical knowledge the nucleus is probably dependent on the cytoplasm for its energy demands. The oxidative phosphorylation of the mitochondria is regarded as one of the main sources of readily utilisable energy in the cell and the uncoupling of this process, which has been found in spleen mitochondria after total body irradiation (Potter and Bethel, 1952; van Bekkum, Jongepier, Nieuwerkerk and Cohen, 1953; Maxwell and Ashwell, 1953), may be expected to result in the impairment of various energy-requiring vital cell functions. More specifically the question arises whether the inhibition of mitosis and of DNA synthesis results from the impairment of phosphorylation or from some other, more fundamental radiation damage. The data so far available are insufficient to allow a conclusion. Potter and Bethel (1952) state that a depression of phosphorylation in isolated spleen mitochondria could be observed 1 hour after total body X-irradiation with a dose of 800 R, but no details are provided. The experiments of Maxwell and Ashwell (1953) were performed on mice at 1–7 days after a lethal dose of X-irradiation. van Bekkum, Jongepier, Nieuwerkerk and Cohen (1954) reported a marked decrease of phosphorylation of isolated rat spleen mitochondria 4 hours after a total body X-ray dose of 1100 R. Clearly the rather large doses of radiation employed, as well as the length of the interval between irradiation and analysis used by the above authors, make it impossible to compare the radiosensitivity of oxidative phosphorylation and of the nuclear functions. It was felt that more

* Stationed by the Surgeon-General of the Royal Netherlands Army at the Medical Biological Laboratory.

information on the effect of dose and time was needed and we have also examined the tissues histologically to allow a direct comparison between the biochemical data and the morphological changes in the cell nuclei. In addition to the spleen we have also examined the thymus to obtain information on a more homogeneous lymphoid tissue.

MATERIALS AND METHODS.

Animals.

Young adult male albino rats were used. The body wt. varied between 200 and 300 g.; corresponding control and irradiated groups of rats were weight-paired. Water and food were provided *ad lib.* until the moment of irradiation, at which time food was withdrawn.

Irradiation technique.

The rats were irradiated in a circular perspex box containing 4 rats, under conditions of maximum backscatter. The physical factors of the radiation were: 200 kV, 20 mA, total filtration 1·5 mm. Cu, H.V.L.: 1·8 mm. Cu, distance to target 50 cm., dose rate 45 R/min.

Oxidative phosphorylation.

The rats were killed by stunning and the required organs were quickly removed and stored in ice-cold 0·25 M sucrose solution. After trimming and weighing, the tissues were homogenised in an all-glass Potter-Elvehjem apparatus. Usually the pooled organs of 2–4 rats were used for the isolation of the mitochondria. In each experiment a control group was included and carried simultaneously through all procedures.

The homogenate was centrifuged at 600 *g.* for 10 min. and the supernatant was centrifuged at 3500 g. for 15 min. The sediment obtained was washed twice by re-suspension and centrifugation at the same speed for 15 min. The fluffy layer was discarded. When the combined supernatant fractions were centrifuged at 7000 g. for 15 min., additional mitochondrial material was sedimented but this preparation yielded lower phosphorylating values than the fraction collected at 3500 g. The values presented in this study always refer to the latter mitochondrial fraction. The mitochondria were suspended in 0·25 M sucrose; the N content of these suspensions varied between 200 and 400 μg./ml. These procedures were carried out in the cold room at 2° using ice-cold solutions.

The incubation medium had the following composition: phosphate buffer, pH 7·4: 0·015 M; succinate: 0·01 M; adenylic acid: 0·001 M; glucose: 0·033 M; hexokinase (purified to step 3A of Berger, Stein, Colowick and Cori (1946)): 0·010–0·015 ml.; $MgCl_2$: 0·007 M; cytochrome *c*: 15×10^{-6} M; NaF: 0·0055 M; ethylenediamine tetra-acetate: 0·003 M; sucrose: 0·08 M; mitochondria: 200–400 μg. N; total volume: 3 ml. Incubation was carried out in O_2 at 38° for 24 min., including an equilibration period of 9 min. Oxygen uptake was estimated during the second 9-min. period and during the last 6-min. period of incubation. The first value was taken to be roughly equal to the O_2 uptake during the equilibration period. The reaction was terminated by the addition of trichloroacetic acid in the cold. Phosphate uptake was measured as the difference between pre- and post-incubation phosphate concentrations with the method of Fiske and SubbaRow (1925). The incubations were performed in duplicate, except in some experiments with thymus, when insufficient material was available. The Kjeldahl method was used for the determination of nitrogen.

Histological techniques.

Samples of the tissues were removed before introduction into the sucrose solution and fixed in Bouin's fluid and in Zenker-formol solution. After fixation the tissue was embedded in paraffin and sections were stained with haematoxylin and eosin and with May-Grünwald Giemsa stain.

RESULTS.

Variation of the Radiation Dose.

The effect of a total body dose of X-radiation of 1100 R has been studied previously at 4 hr. after the irradiation. This interval was adhered to in the present

experiments and a series of smaller radiation doses was first investigated to obtain some insight into the radiosensitivity of oxidative phosphorylation. Table I shows that a sharp decrease of phosphorylation was found even after doses of 300 R. Therefore in the next series of experiments, on which the histological

TABLE I.—*Effect of Various Doses of Total Body Irradiation on the Oxidative Phosphorylation of Isolated Spleen Mitochondria.*

R administered 4 hr. before analysis.	Number of experiments.	Phosphate uptake.*	Oxygen uptake.*	P/O ratio.*
900	3	41	78	53
700	6	54	75	74
500	5	66	84	77
300	3	57	75	80

* Percentage of corresponding control value.

observations were made, smaller doses have been included. The details of the latter experiments are presented in Table II. In spleen mitochondria a distinct depression of phosphate uptake has been observed after the 100 R doses, while the 50 R doses had no effect. In the case of thymus mitochondria however a decreased

---

EXPLANATION OF PLATES.

All photographs are of paraffin sections made from thymus and spleen tissue of rats, 4 hr. after a total body X-irradiation. Fixation Bouin, haematoxylin and eosin stained. × 510.

FIG. 1.—Thymus, control.

FIG. 2.—Thymus, 4 hr. after 50 R. Shows some pyknotic and fragmented nuclei.

FIG. 3.—Thymus, 4 hr. after 100 R. Shows a great number of pyknotic and fragmented nuclei.

FIG. 4.—Thymus, 4 hr. after 300 R. Shows severe pyknosis and fragmentation of nuclei and nuclear vacuolisation.

FIG. 5.—Spleen, control.

FIG. 6.—Spleen, 4 hr. after 50 R. Shows some pyknotic and fragmented nuclei and a few nuclear vacuoles.

FIG. 7.—Spleen, 4 hr. after 100 R. Shows a great number of pyknotic and fragmented nuclei and some nuclear vacuoles.

FIG. 8.—Spleen, 4 hr. after 300 R. Shows severe pyknosis and fragmentation of nuclei.

All photographs are of paraffin sections made from spleens of rats at different times after a total body X-irradiation with a dose of 700 R. Fixation Bouin; haematoxylin and eosin stained. × 790.

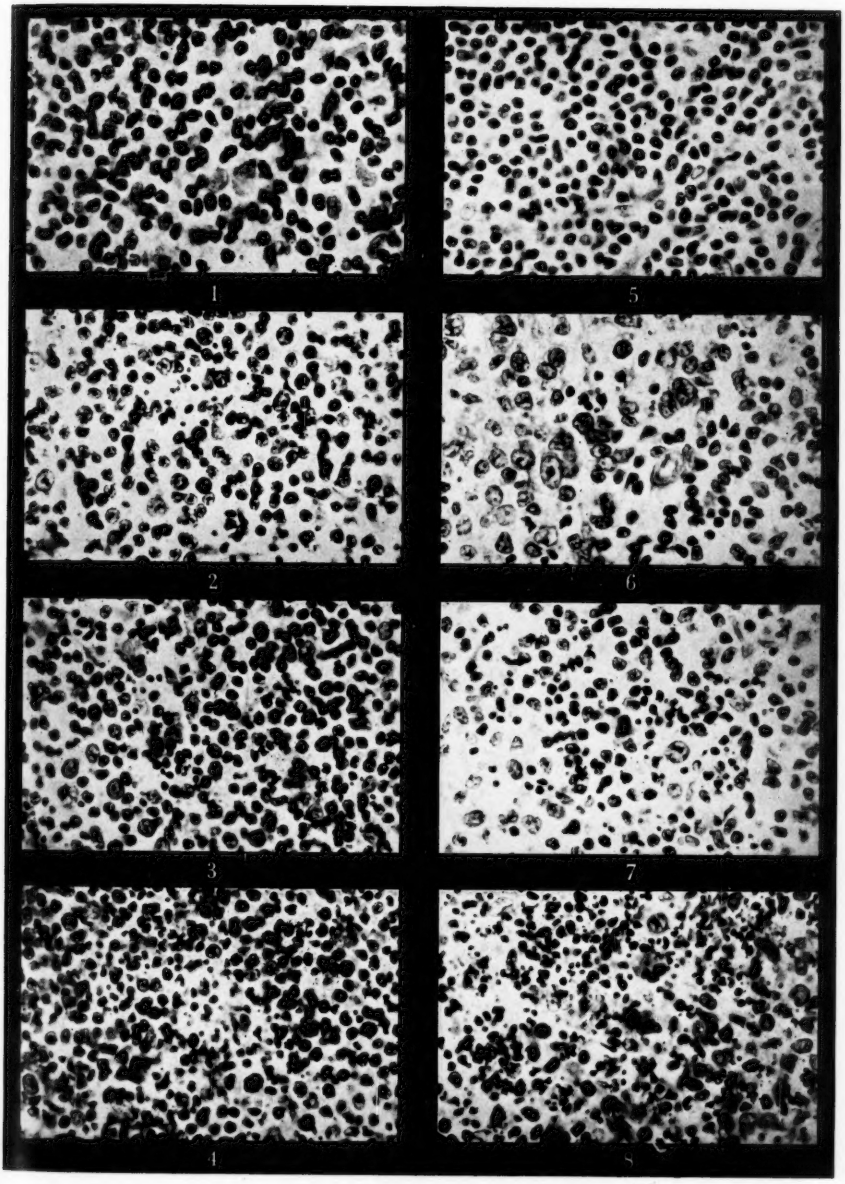
FIG. 9.—Spleen, control.

FIG. 10.—Spleen, ½ hr. after irradiation. Nuclear degeneration not apparent.

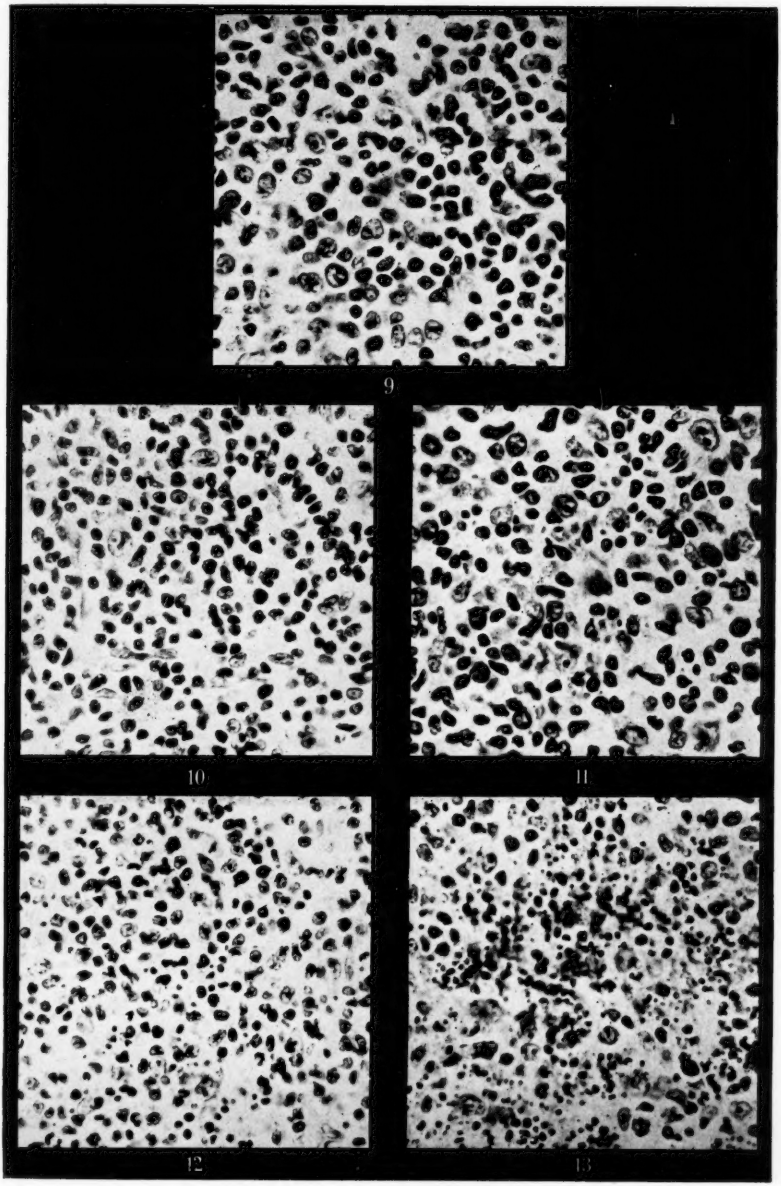
FIG. 11.—Spleen, 1 hr. after irradiation. Shows nuclear vacuoles, pyknosis and beginning nuclear fragmentation.

FIG. 12.—Spleen, 2 hr. after irradiation. Shows nuclear vacuoles, pyknosis and nuclear fragmentation.

FIG. 13.—Spleen, 4 hr. after irradiation. Shows severe pyknosis and nuclear fragmentation in the majority of cells. Very few nuclear vacuoles are present.



van Bekkum and Vos.



van Bekkum and Vos.

TABLE II.—*Oxidative Phosphorylation of Isolated Spleen Mitochondria 4 Hours after Total Body X-Irradiation.*

Treatment.	Phosphate uptake μ mol./mg.N.	Oxygen uptake μ atom/mg.N.	P/O ratio.
Controls	26·2	24·2	1·09
500 R	14·6	16·9	0·86
Controls	33·7	35·7	0·94
300 R	17·0	25·5	0·67
Controls	35·8	36·0	1·00
100 R	21·0	27·8	0·76
Controls	24·0	25·8	0·93
100 R	15·5	23·0	0·67
Controls	36·0	28·3	1·28
50 R	38·0	31·6	1·20
Controls	29·6	33·8	0·88
50 R	26·9	33·2	0·81

phosphorylation was apparent after a dose of 50 R (Table III). Although only 2 experiments were performed it should be noted that each value was obtained from the pooled thymus tissue of 4 rats. Generally lower P/O ratios were obtained with thymus mitochondria than with spleen mitochondria. The fact that thymus mitochondria are predominantly derived from lymphocytes, while spleen mitochondria probably represent a mixture of mitochondria from various cell types, may be the cause of this discrepancy.

TABLE III.—*Oxidative Phosphorylation of Isolated Thymus Mitochondria 4 Hours after Total Body X-Irradiation.*

Treatment.	Phosphate uptake μ mol./mg.N.	Oxygen uptake μ atom/mg.N.	P/O ratio.
Controls	17·0	32·7	0·52
500 R	6·9	25·0	0·28
Controls	21·9	57·0	0·38
300 R	9·8	36·3	0·27
Controls	18·0	50·1	0·36
100 R	15·7	35·7	0·44
Controls	23·1	39·9	0·58
100 R	15·9	28·3	0·56
Controls	14·8	43·2	0·34
50 R	5·8	39·7	0·15
Controls	10·9	36·0	0·30
50 R	8·3	29·7	0·28

The corresponding histological sections showed definite changes at 4 hr. after the lowest dose (50 R) in both thymus and spleen (Fig. 2 and 6). These changes, which consisted of pyknosis, fragmentation and vacuolisation of the nuclei of a small number of lymphocytes were assessed quantitatively by counting 2000 cells in preparations of the two experiments with thymus. The percentage of

degenerated nuclei was 1·0 and 1·7 in the control preparations and 3·7 and 8·9 in the 50 R series. The number of dividing nuclei was 7 per 1000 in the controls and 4 per 1000 in the 50 R group. The damage in the spleen of the 50 R group was almost exclusively limited to the lymph follicles and the amount of degenerated nuclei therein was comparable to that observed in the thymus preparations. The fact that rat spleen is only partly made up of lymphoid cells suggests that the slightly greater radiosensitivity of the thymus mitochondria might be due to the greater radiosensitivity of lymphocytes as compared to other haematopoietic cells. After the dosages of 100 R the number of lymphocyte nuclei in some stage of degeneration was far greater and fragmentation was more frequently present (Fig. 3 and 7).

After 300 R nuclear degeneration in these lymphoid tissues dominated the picture (Fig. 4 and 8) and after the larger doses an additional small increase occurred in the number of pyknotic nuclei. A reasonable estimation of the radiation dose received by microscopical inspection proved possible up to the 300 R dosage. Above 300 R it was difficult to assess a difference of 200 R because the relative increase of nuclear damage was small, compared with the individual variation. From the results obtained with thymus and spleen mitochondria after the smallest doses, *viz.*, 50 R and 100 R, it is concluded that the oxidative phosphorylation in these organs represents at least one cytoplasmic function, the radiosensitivity of which is comparable to that of the cell nucleus.

Variation of the Post-irradiation Period.

These experiments were performed on spleen tissue after a total body irradiation with a dose of 700 R. This rather large dose was chosen because it always produced a sharp drop of phosphorylation after an interval of 4 hr. The post-irradiation periods are shown in Table IV and the values of the oxidative phosphorylation are presented as percentages of the corresponding control data. A significant depression of phosphate uptake was found 2 hr. after irradiation and a highly significant decrease after the 4-hr. period. One hr. or less after irradiation effects are either absent or so small as to require many more experiments to show up significantly.

TABLE IV.—*Oxidative Phosphorylation of Isolated Spleen Mitochondria at Various Intervals after Total Body X-Irradiation.*

Hours after irradiation with 700 R.	Number of experiments.	Phosphate uptake.*	Oxygen uptake.*	P/O ratio.*
¼	5	107 ± 24	99 ± 7	107 ± 20
½	3	93 ± 5	100 ± 4	93 ± 5
1	4	93 ± 8	98 ± 3	96 ± 10
2	8	75 ± 7†	90 ± 4	82 ± 7
4	8	54 ± 6‡	77 ± 4	72 ± 6

* Percentage of corresponding control mean ± S.E. values.
† Mean decrease of phosphate uptake 25 ± 7 per cent, $t = 3 \cdot 55$, $P < 0 \cdot 01$.
‡ Mean decrease of phosphate uptake 46 ± 6 per cent, $t = 7 \cdot 30$, $P < 0 \cdot 001$.

The histological findings are summarised in Table V. As early as 15 min. after irradiation, the number of mitoses had decreased so much that preparations could be easily differentiated from control sections on this criterion only. Signs of

nuclear degeneration were absent at this stage as well as 30 min. after irradiation (Fig. 10). One hr. after irradiation nuclear degeneration became clearly discernible. The nuclear changes were limited to a few cells only and consisted mainly of vacuolisation. In addition some nuclei showed pyknosis and the beginning of fragmentation (Fig. 11). After 2 hr. nuclear degeneration was present in 10–20 per cent of the lymphocytes (Fig. 12) and after 4 hr. pyknosis and nuclear fragmentation were present in the majority of the cells (Fig. 13).

TABLE V.—*Morphological Effects of* 700 R *of Total Body X-Irradiation on Spleen Tissue at Various Intervals after Irradiation.*

Interval.	¼ hour.	½ hour.	1 hour.	2 hours.	4 hours.
Number of mitoses	Greatly diminished	Greatly diminished	Greatly diminished	Greatly diminished	Still less than in controls.
Nuclear degeneration	None	Absent or dubious	Present in a small number of cells. Predominantly vacuolisation, some pyknosis and beginning fragmentation	Pyknosis, fragmentation and all earlier stages of degeneration in 10–20 per cent of lymphoid cells	All stages of degeneration in majority of lymphocytes, some phagocytosis.

The amount of nuclear degeneration in our preparations is in fair agreement with the results of Trowell (1952) on rat lymph glands after *in vivo* irradiation with 400 R. This author counted the degenerated nuclei 1, 2, 3 and 4 hr. after irradiation and found respectively 2, 14, 49 and 78 per cent degenerated nuclei. The coagulation of chromatin in the nucleus which precedes the vacuolisation according to Trowell, could not be recognised with certainty in our experiments. Therefore vacuolisation was considered as the first definite sign of nuclear degeneration. It is of interest that the various stages of degeneration did not develop simultaneously in all lymphocytes. For instance after 4 hr. normal cells were observed among others showing all stages of degeneration, including fragmentation. No changes were observed in the reticulo-endothelial cells and active phagocytosis of nuclear fragments appeared at 4 hr. after irradiation. After 12 hr. most of the débris was taken up by macrophages and 24 hr. after irradiation the nuclear fragments were completely removed. In former experiments it was found that 24 hr. after irradiation the oxidative phosphorylation is still at a very low level (van Bekkum *et al.*, 1954).

DISCUSSION.

Since it has been shown that the oxidative phosphorylation of spleen and thymus mitochondria is a highly radiosensitive process, the significance of its disturbance with regard to the development of nuclear degeneration becomes of interest.

From the experiments with small doses of total body irradiation it is concluded that the radiosensitivities of the nucleus and of the mitochondria of the tissues studied do not differ widely, if at all. The results obtained using various post-irradiation periods show that signs of nuclear damage can be observed at an earlier time after irradiation than a decrease of oxidative phosphorylation. This, however, cannot be taken as proof that nuclear degeneration precedes the disturbance of the mitochondrial functions. While microscopical inspection

allows the detection of changes in a single cell, the biochemical method used is far less sensitive because the mitochondria are derived randomly from all cells. Therefore a comparison can at present be made only between the total number of damaged nuclei and the overall change of oxidative phosphorylation. Changes of a few per cent in the latter will not be detectable owing to the considerable variation that has been encountered in control rats. Taking this into account, it may be concluded that the development of nuclear damage and the disturbance of mitochondrial function after irradiation run roughly parallel. The decision whether a primary rôle in the development of radiation-induced cell damage should be attributed to one of these aberrations has to await further investigation. On the other hand, the possibility remains that both the nuclear and the mitochondrial defects represent a non-specific result of some other as yet unknown disturbance of the cellular metabolism.

SUMMARY.

Rats were exposed to various dosages of X-rays and the effect on the oxidative phosphorylation of the isolated spleen and thymus mitochondria was investigated. This process was found to be very radiosensitive. Oxidative phosphorylation was depressed in the thymus after doses as small as 50 R and in the spleen after 100 R.

Variation of the post-irradiation period showed that a significant depression of the phosphorylation of isolated rat spleen mitochondria develops as early as 2 hours after irradiation with a total body dose of 700 R.

The tissues used were subjected to histological examination in order to obtain information on the possible relation between mitochondrial and nuclear damage. It was found that these effects run roughly parallel and it has not been possible to deduce a causal relationship between the two phenomena.

The authors acknowledge gratefully the skilled technical assistance of Miss H. T. M. Nieuwerkerk and Miss L. Budke. Our thanks are due to the Surgeon General of the Royal Netherlands Army for the assignment of one of us (O. V.) to this laboratory.

REFERENCES.

BERGER, L., STEIN, M. W., COLOWICK, S. P. AND CORI, C. F.—(1946) *J. gen. Physiol.*, **29**, 379.
FISKE, C. H. AND SUBBAROW, Y.—(1925) *J. biol. Chem.*, **66**, 375.
HIRSCH, G. C.—(1931) *Arch. EntwMech. Org.*, **123**, 792.
LUDFORD, R. J.—(1932) *10th Sci. Rep. Cancer Res. Fd, Lond.*, p. 126.
MAXWELL, E. AND ASHWELL, G.—(1953) *Arch. Biochem.*, **43**, 389.
NADSON, G. A. AND ROCHLIN, E. J.—(1934) *Protoplasma*, **20**, 31.
POTTER, R. L. AND BETHEL, F. H.—(1952) *Fed. Proc.*, **11**, 270.
TROWELL, O. A.—(1952) *J. Path. Bact.*, **64**, 687.
VAN BEKKUM, D. W , JONGEPIER, H. J., NIEUWERKERK, H. T. M. AND COHEN, J.A.—(1953) *Trans. Faraday Soc.*, **49**, 329—(1954) *Brit. J. Radiol.*, **27**, 127.

SURVEY OF PAPERS

SHOOTER and WYATT have studied the mineral requirements for growth of *Staphylococcus pyogenes*, creating a deficient medium by passing nutrient broth through an ion exchange column. They find that magnesium and calcium are necessary, and suggest that calcium and possibly potassium are required for the formation of staphylococcal proteases (p. 341).

LONG and JENNIFER SHEWELL have studied the influence of the thryoid gland on the production of diphtheria antitoxin in guinea-pigs. They find that thyroxine increases immunity and that this is prevented by partial pancreatectomy. They conclude that thyroxine increases immunity by inducing compensatory hypertrophy in the pancreas, presumably of the islets of Langerhans (p. 351).

ROBERTS has estimated agglutinin titres in extracts of omentum, of other organs and in serum after intraperitoneal injections of killed *Salmonella* : in some experiments a different species was injected simultaneously into the blood stream. From immunised animals, living or heat-killed omental tissue was transferred to normal rabbits. The results suggest that the omentum can be a site of antibody formation (p. 357).

GLADSTONE and JOHNSTON have found certain strains of *B. anthracis* to be highly susceptible to the action of lysozyme when cultured in 10–20 per cent CO_2 in a medium containing 0·05–0·1 M-HCO'_3. None of the strains tested was susceptible when grown in air, and the phenomenon does not appear to depend on capsulation of the bacilli or to be related to their virulence or power to produce protective antigen (p. 363).

VAN HEYNINGEN has studied the effect of iron, cobalt and nickel on the growth, respiration and toxin production of *Sh. shigae*, and on the growth and respiration of *Sh. flexneri*, *Sh. sonnei* and *Bact. coli*. Observations of general interest have been made, although the mechanism of toxin production is, so far, obscure (p. 373).

VAN HEYNINGEN, in continuation of the work described in the preceding paper, has investigated the relation between toxin and cytochrome production by *Sh. shigae*. He finds that the toxin production is dependent on efficient haem formation, and suggests that for *Sh. shigae* it is controlled by a number of factors, only some of which are understood (p. 381).

EVANS and PERKINS, in a continuation of their studies of immunity in mice to infection with *H. pertussis*, have shown that the early immunity following a single injection of pertussis vaccine is transient, and is quite distinct from an immunity, dependent on antibody formation, which develops more slowly and reaches its peak at a time when the initial " interference " immunity has faded. (p. 391).

LOGOTHETOPOULOS and WEINBREN found abundant protein droplets in the proximal renal tubules of male rats aged 60 days or older. They were able to correlate this phenomenon with the level of proteinuria. In female rats only few inclusions were found and proteinuria was less than in male rats. On injection of testosterone the level of proteinuria rose but not the numbers of inclusions (p. 402).

HOLT and JOAN M. BARNES have analysed mathematically the factors involved in the different dose-responses of guinea-pigs to diphtheria toxoid and to P.T.A.P. Within a particular dose range an antigenicity equation is applicable and in these conditions an accurate determination of the potency of one antigen in terms of the other can be made (p. 407).

JOAN M. BARNES and HOLT find that in guinea-pigs added pertussis vaccine enhances the antibody response to diphtheria toxoid by increasing the efficiency of the primary stimulus. An inverse correlation was shown between the size of the dose inoculated and the magnitude of the adjuvant effect (p. 415).

KNOX and WOODROFFE added isoniazid to haemin and found that an isoniazid haemochromogen was formed which was stable in the absence of molecular oxygen. In the presence of air isoniazid was oxidised, haemin acting as a catalyst of the reaction. The addition of haemin to isoniazid-containing medium reversed the inhibition of growth of *Myco. tuberculosis*, but this addition was fully effective only if made within a few hours of inoculation (p. 425).

VAN BEKKUM and VOS exposed rats to X-rays and determined the decrease in oxidative phosphorylation of the isolated mitochondria from spleen and thymus. They also examined these organs histologically and found that the development of nuclear damage runs roughly parallel to that of the disturbance of mitochondrial function (p. 432).

EXCERPTA MEDICA
International Abstract Service
Covering all fields of Clinical
and Experimental Medicine

WILLIAM BOYD, M.D., Toronto:
"— This is a feature of the greatest value to the pathologist who wishes to keep abreast of the advances in his subject throughout the world. . . ."

A Complete Survey

of all available medical journals in the world—abstracted exhaustively, accurately—on morphological and experimental pathology and related subjects of interest to the pathologist.

GENERAL PATHOLOGY AND PATHOLOGICAL ANATOMY

Section V of Excerpta Medica

Subscription rate £9 6 0 yearly or the equivalent in local currency

Free prospectus and specimen copy on demand

In this organisation a global fraternity of about 5,000 medical specialists co-operate to serve medical science.

Distributors all over the world.

Central Office: Excerpta Medica, 111 E Kalverstraat, Amsterdam, Netherlands.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXXVI, No. 4. AUGUST, 1955.

CONTENTS.

PAGE

SHOOTER, R. A. AND WYATT, H. V.—MINERAL REQUIREMENTS FOR GROWTH OF *Staphylococcus pyogenes*. EFFECT OF MAGNESIUM AND CALCIUM IONS . 341

LONG, D. A. AND SHEWELL, JENNIFER—THE INFLUENCE OF THE THYROID GLAND ON THE PRODUCTION OF ANTITOXIN IN THE GUINEA-PIG 351

ROBERTS, K. B.—ANTIBODY FORMATION IN THE OMENTUM 357

GLADSTONE, G. P. AND JOHNSTON, H. H.—THE EFFECT OF CULTURAL CONDITIONS ON THE SUSCEPTIBILITY OF *Bacillus anthracis* TO LYSOZYME 363

VAN HEYNINGEN, W. E.—THE NEUROTOXIN OF *Shigella shigae*. 5. THE EFFECT OF IRON AND RELATED METALS ON THE GROWTH, RESPIRATION AND TOXIN PRODUCTION OF *Shigella shigae* AND RELATED ORGANISMS 373

VAN HEYNINGEN, W. E.—THE NEUROTOXIN OF *Shigella shigae*. 6. THE RELATION BETWEEN TOXIN AND CYTOCHROME PRODUCTION IN THE ORGANISM . . 381

EVANS, D. G. AND PERKINS, F. T.—THE PRODUCTION OF BOTH INTERFERENCE AND ANTIBODY IMMUNITY BY PERTUSSIS VACCINE TO PERTUSSIS INFECTION IN MICE 391

LOGOTHETOPOULOS, J. AND WEINBREN, K.—NATURALLY OCCURRING PROTEIN DROPLETS IN THE PROXIMAL TUBULE OF THE RAT'S KIDNEY 402

HOLT, L. B. AND BARNES, JOAN M.—QUANTITATIVE STUDIES IN DIPHTHERIA PROPHYLAXIS: THE DETERMINATION OF THE ANTIGENICITY CHARACTERISTICS OF PURIFIED DIPHTHERIA TOXOID IN SIMPLE SOLUTION AND AS P.T.A.P. 407

BARNES, JOAN M. AND HOLT, L. B.—QUANTITATIVE STUDIES IN DIPHTHERIA PROPHYLAXIS. ANTIGENIC INTERACTIONS: I. SIMULTANEOUS INOCULATIONS 415

KNOX, R. AND WOODROFFE, R. C.—HAEMIN-ISONIAZID INTERACTION AND THE EFFECT OF HAEMIN IN "REVIVING" ISONIAZID-TREATED TUBERCLE BACILLI 425

VAN BEKKUM, D. W. AND VOS, O.—THE EFFECT OF X-RAYS ON THE OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA IN RELATION TO NUCLEAR DAMAGE 432

SURVEY OF PAPERS . . . . . (i)

PRINTED BY ADLARD AND SON, LIMITED, BARTHOLOMEW PRESS, DORKING.